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PATENT & TRADEMARK OFFICE

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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicants:	Akihiro FUNAKOSHI *et al.*
Title:	A PREVENTIVE OR THERAPEUTIC AGENT FOR PANCREATITIS COMPRISING IL-6 ANTAGONIST AS AN ACTIVE INGREDIENT
Appl. No.:	09/762,550
International Filing Date:	8/23/1999
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Examiner:	Lorraine Spector
Art Unit:	1647
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REPLY UNDER 37 C.F.R § 1.116

Mail Stop AF
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Sir:

This communication is responsive to the Final Office Action dated December 15, 2008, concerning the above-referenced patent application. Enclosed are a Petition for Extension of Time, six (6) exhibits (A-F); and a Notice of Appeal. Applicants respectfully request entry and consideration of the attached exhibits and remarks.

I. Status of the claims

No claims are newly amended, added or cancelled. Claims 1-13, 15, 24 and 27-39 were previously cancelled and claims 14, 16-23, 25 and 26 are pending in the application.

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II. Rejections under 35 U.S.C. §103(a)

At pages 2-6 of the Office Action, the Office finds unpersuasive Applicants' remarks and reasserts that all claims are allegedly obvious over Sato *et al.*, *Cancer Research* 53(4):851-856 (1993) ("Sato"), and/or Kishimoto *et al.*, EP 0 791 359 A1 ("Kishimoto"), either or both references in view of Gross *et al.*, *Hepato-Gastroenterol.* 40:522-530 (1993) ("Gross"), and Farkas *et al.*, *Neuroscience Letters* 242(3):147-150 (1998) ("Farkas"). Applicants respectfully traverse, for reasons of record and further in view of the following comments and Exhibits A-F.

A. The prior art evidence for IL-6 as a causative agent of pancreatitis

The Office asserts the claims are obvious because (a) IL-6 was a known inflammatory cytokine and (b) IL-6 was known to be associated with pancreatitis. Applicants do not dispute (a) or (b), but they are *incomplete* and gloss over important facets of the knowledge available at the time of filing. A determination of obviousness requires consideration of the "subject matter as a whole" (35 U.S.C. § 103(a)), and such a consideration leads to a finding of nonobviousness.

First, while IL-6 is known to be pro-inflammatory, it also known to have important ***anti-inflammatory*** properties. *See e.g.,* Exhibit A, wikipedia entry on IL-6 "Interleukin-6 (IL-6) is an interleukin that acts as both a pro-inflammatory and anti-inflammatory cytokine" (en.wikipedia.org/wiki/ interleukin_6, accessed May 9, 2009). The anti-inflammatory properties of IL-6 were known well before the filing date of the present application, and had been observed both in *vitro* and *in vivo,* especially as part of a negative feedback loop with TNFα. Such negative feedback systems are extremely common and well known in biology. *See, e.g.* Exhibit B, en.wikipedia.org/wiki /negative_feedback, accessed May 9, 2009.

Publishing in the highly rated Journal of Immunology, Aderka *et al.* states in 1989 that "[i]nasmuch as TNF is known to be an inducer of IL-6, the inhibitory action of IL-6 on TNF production may represent the negative arm of a regulatory circuit. The inhibitory action of IL-6 on TNF production is consistent with a predominantly antiinflammatory role of IL-6 in the intact organism." *See* Exhibit C, Aderka *et al.* "IL-6 inhibits lipopolysaccharide-induced

tumor necrosis factor production in cultured human monocytes, U937 cells, and in mice." *J. Immunol.* 143(11):3517-3523 (1989) ("Aderka"). Aderka's notes that both IL-1β and TNFα stimulate IL-6, but that IL-6 inhibits only TNFα, apparently at the level of TNFα synthesis. *Id.* at 3522, col. 2.

Aderka further notes that the TNFα inhibitory effect of IL-6 is consistent with many other anti-inflammatory properties of IL-6.

> IL-6 is a major inducer of the acute phase response mediating the synthesis and release of CRP, α_2-M, α_1-PI, and other acute phase proteins (5). CRP is selectively deposited on necrotic cells that have phospholipids exposed on their damaged membranes (32). In addition. CRP may form complexes with cellular toxic constituents, such as chromatin fragments released into the circulation as a result of tissue damage, thereby perhaps facilitating their clearance (33). ***The function of α_2-M and α_1-PI is thought to be mainly antiinflammatory*** as these proteins normally form a protective barrier between tissue and elastase-the latter being the most potent proteolytic enzyme released by activated neutrophils. TNF, released in septic conditions (34), may activate and prime neutrophils to release oxygen radicals (35). These radicals oxidize and irreversibly inactivate α_2-M and α_1-PI thus exposing tissues to the digestive attack by the neutrophil elastase (36). ***By stimulating the acute phase response of the liver, IL-6 is triggering a process whose major function may be to indirectly counterbalance the toxic actions of TNF and other mediators of inflammation. Here we identified an additional potentially beneficial action of IL-6, i.e. its ability to reduce TNF production and thereby reduce the total inflammatory burden. Furthermore. because TNF cytotoxicity may be partly dependent on protease activity (37), IL-6-induced production of protease inhibitors by the liver may, theoretically, moderate TNF cytotoxicity.***
>
> ***Hepatocyte stimulating factor/IL-6 was also reported to induce ACTH release (38) and, consequently, cortisol — the best known antiinflammatory agent.***

Id. at page 3522, col. 2., emphasis added.

Consistent with Aderka, *id.*, Ulich *et al.* states in the abstract "[i]nterleukin-6 also is shown to be endogenously upregulated within the lung after intratracheal challenge with

endotoxin, providing evidence that IL-6 may represent an endogenous negative feedback mechanism to inhibit endotoxin-initiated cytokine-mediated acute inflammation " *See* Exhibit D, Ulich *et al.* "Intratracheal injection of endotoxin and cytokines" *Amer. J. Pathol.*, 138(5):1097-1101 (1991) ("Ulich"). Ulich showed that LPS injection caused an increase in IL-6 expression: "Intratracheal injection of LPS caused IL-6 mRNA expression beginning at 2 hours and increasing markedly at 4 hours (Figure 1)." (*id.*, p1099, col. 1). By the logic of the Office Action, this would indicate IL-6 acting as a pro-inflammatory cytokine – but Ulich demonstrated that the *reverse* was true: IL-6 was *anti-inflammatory*. LPS induced more inflammation alone than IL-6 and LPS together. The reason that IL-6 rose in response to inflammation was not because it was pro-inflammatory, but because it was an *anti-inflammatory* component of an endogenous negative feedback loop to *counter* the pro-inflammatory effects of TNFα. Ulich concludes, in the discussion, "IL-6 and TGFβ, alone and especially in combination, are shown to inhibit strongly LPS-induced acute inflammation" (*id.*, p1011, col. 1), and:

> The mechanism of the anti-inflammatory action of IL-6 and TGFβ may relate to the ability of these cytokines to inhibit TNFα (and as shown by the present data) and IL-1 production by macrophages. Host-derived IL-6 is upregulated locally after challenge with LPS and may act as an endogenous negative feedback mechanism to inhibit the LPS-initiated 1L-l and TNF-mediated acute inflammatory process

(*id.*, p1100, col. 2.).

Ulich demonstrates that the observed correlation between IL-6 and inflammation does *not* indicate that IL-6 is proinflammatory and, in fact, indicates that IL-6 is acting as an *anti-inflammatory* cytokine. If IL-6 is acting as an anti-inflammatory cytokine as part of an endogenous negative feedback loop, the person of ordinary skill would expect that IL-6 would occur *later* in inflammation, rather than earlier. Considering the subject matter as a whole, including Aderka and Ulich, *id.*, the person of ordinary skill would consider that *timing* is at least as important as the level of cytokine, with a delayed rise in IL-6 levels indicating an anti-inflammatory effect.

Experimental pancreatitis models demonstrate that IL-6 is induced late, consistent with an anti-inflammatory action. For example, Norman *et al.* report that "[t]umor necrosis factor-α (TNF-α) and interleukin-1-β (IL-1β) mRNA and protein were detected within the pancreas early in the course of pancreatitis in both models, coinciding with the development of hyperamylasemia (both $p<0.001$). Interleukin-6 was produced in the pancreas *after* pancreatitis was more fully developed ($p<0.00l$)." *See* abstract of Exhibit E, Norman *et al.*, "Tissue-specific cytokine production during experimental acute pancreatitis" *Digestive Diseases and Sciences* 42(8): 1783 – 1788 (1997) ("Norman") Moreover, "[t]he delayed expression of IL-6 mRNA within the pancreatic parenchyma is consistent with the previously demonstrated pattern of delayed IL-6 elevation in the serum during experimental and clinical pancreatitis (5-9)." Norman, *id.*, p. 1786, col. 1.

The same delay in IL-6 levels is also observed in art that the Office alleges demonstrates that IL-6 is pro-inflammatory, but which is more consistent with anti-inflammatory action. Thus, Farkas' observation that "induction of TNF preceded that of IL-6, and the peak of serum TNF level was seen in the first 24h during which the increased blood-brain barrier permeability was detected" is entirely consistent with a *negative* regulator of inflammation and with Ulich and Aderka, *id.*.

As to Gross's observation that IL-6 levels are predictive of the severity of pancreatitis, this too is unsurprising from the perspective of IL-6 as an anti-inflammatory cytokine – the pro-inflammatory cytokines TNFα and IL-1 β are known to induce IL-6 (*see* Aderka, Ulich, *id.*) and so high levels of these proinflammatory mediators would be expected to induce more IL-6 to mitigate excessive inflammation by (a) directly inhibiting TNFα synthesis; (b) induction of α_2-M and α_1-PI to inhibit proteolysis; (c) stimulating production of CRP to assist in clearance of damaged tissue and; (d) stimulation of cortisol release.

Thus:

(a) IL-6 is known to have both pro- and anti-inflammatory activities

(b) IL-6 is induced by TNFα and IL-1β and is known to directly inhibit TNFα and induce the production of anti-inflammatory factors and

(c) is consistently observed later in pancreatitis, *following* the rise in TNFα and pancreatic inflammation.

The evidence points towards IL-6 acting as an *anti-inflammatory* cytokine induced as part of the negative feedback to TNFα, and not causing inflammation. In such a situation, the administration of IL-6 antagonists would be expected to prevent the downregulation of TNFα, prevent the production of protective factors, and thereby *exacerbate*, not treat, acute pancreatitis. The logical target is TNFα, oreven IL-1, not IL-6. *See e.g.* Grewal *et al.* "Amelioration of the physiologic and biochemical changes of acute pancreatitis using an anti-TNF-alpha polyclonal antibody" *Am. J Surg.* 167(1):214-218 (1994), Exhibit F, reporting amelioration of pancreatitis by inhibiting polyclonal antibodies against TNFα.

Accordingly, the person of ordinary skill would believe that the evidence pointed to IL-6 as an anti-inflammatory cytokine in acute pancreatitis.

Applicants note that page 3 of the Office Action states:

> applicants argue that if there were sufficient evidence of a causative link between IL-6 and acute pancreatitis, that such would have been made explicit in the art. This argument has been fully considered but is not deemed persuasive. It is not necessary that there have been an explicit teaching of such; the standard under 35 U.S.C. §103(a) is whether or not the person of ordinary skill in the art would have found the Invention obvious over the cited prior art. The examiner has established a prima facie case of obviousness; the cited art clearly indicates IL-6 to be a likely causative agent of acute pancreatitis.

Applicants do not deny that a *prima facie* case can exist without an explicit teaching. However the very notable lack of any explicit teaching is, clearly, at odds with the Office's position that the art was so abundantly clear, suggesting an impermissible hindsight reconstruction of the data.

B. No motivation

Page 3 of the Office Action states:

> Further, even if it were merely associated with, and not a causative agent of acute pancreatitis, the person of ordinary skill in the art would have been motivated to administer an IL-6 antagonist in view of the fact that IL-6 is an inflammatory cytokine, acute pancreatitis is an inflammatory condition, and the art clearly indicates that IL-6 is elevated in acute pancreatitis. Therefore, applicants arguments as to whether IL-6 is merely causative or associated with the condition are moot.

This argument relies on the incorrect assumption that IL-6 is *only* an inflammatory cytokine, and ignores the very clear teaching away in the prior art and therefore fails to consider the "subject matter as a whole" *See* 35 U.S.C. § 103. Even *if* the art was more equivocal, and the Office is asserting that it would have been obvious to try, this is insufficient to render obvious the claims.

The Supreme Court's decision in *KSR* stated that "obvious to try" required the existence of "a ***finite number*** of ***identified, predictable*** solutions" *KSR Int'l Co. v. Teleflex, Inc.* 127 S.Ct. 1727, 1742 (2007), emphasis added. Thus, to the extent that *KSR* overrules *In re Deuel*'s holding that "obvious to try" does not constitute obviousness," *In re Deuel*, 51 F.3d 1552, 1559 (Fed. Cir. 1995)), it requires (a) a problem to be solved, and (b) a limited number of identified, *predictable* solutions. *KSR*, 127 S. Ct. at 1739, 1742. The Federal Circuit affirmed this analysis in *Eisai Co. v. Dr. Reddy's Laboratories, Inc.*, 533 F.3d 1353 (Fed. Cir. 2008) and noted that "to the extent an art is unpredictable, as the chemical arts often are, KSR's focus on these 'identified, predictable solutions' may present a difficult hurdle because potential solutions are less likely to be genuinely predictable." *Eisai* at 1359

The art suggests IL-6 is an anti-inflammatory cytokine in acute pancreatitis and, even if ambivalent as to its role, that the problem should be solved by antagonists to TNFα and/or IL-1. Consistent with this problem/solution approach is the prior art teaching therapy of acute pancreatitis by administering antagonists to TNFα and/or IL-1. *See, e.g.* Grewal, *id.*

C. Secondary indicia of nonobviousness

Even *if* the Office had demonstrated a *prima facie* case, it would be rebutted by secondary indicia of nonobviousness. Unexpected results and teaching away have been long recognized as a classical secondary indicia of nonobviousness. *See Graham v. John Deere Co.*, 86 S.Ct. 684, 694 (1966); *Custom Accessories v. Jeffrey-Allan Industries*, 807 F.2d 955, 960 (Fed. Cir. 1986); *In re Soni*, 54 F.3d 746, 750 (Fed. Cir. 1995), and such secondary indicia have been recognized as "[o]ne way for a patent applicant to rebut a *prima facie* case of obviousness . . . ." *Soni*, 54 F.3d at 750.

Applicants have already addressed the teaching away found in the art, notably that IL-6 is upregulated by TNFα as part of negative feedback that mitigates the pro-inflammatory effects of TNFα, and that the temporal relationship between TNFα, IL-1 and IL-6 are consistent with IL-6 acting as an *anti*-inflammatory cytokine in pancreatitis. In such a context, that an IL-6 antagonist works to treat acute pancreatitis is contrary to the teachings of the art and an unexpected result.

But this is not the only reason. As previously noted in the Reply of June 22, 2006, the person of ordinary skill in the art understood that the cytokine network is complex and has overlapping redundancies. For example, the IL-6 family of cytokines comprises a number of cytokines, such as IL-11, LIF, OSM, CNTF, CT-1, *etc.*, overlapping biological activities, and, while binding to separate receptors, all require gp130 for signal transduction. *See, e.g.,* Taga, T. & Kishimoto, T., *Annu. Rev. Immunol.*, 15:797-819 (1997) at 799-801 (previously provided). Indeed, the Office voiced this same conclusion, noting that "the person of ordinary skill in the art would not expect that suppression of the effects of a single cytokine would completely stop or prevent a condition that is due to the effects of multiple cytokines." Office Action dated October 6, 2003, pg. 3, ln. 6-10.

In view of this redundancy, inhibition of IL-6 alone cannot be predicted to prevent signal transduction. *Even if* IL-6 was known to be a proinflammatory cytokine in acute pancreatitis, it would not have been predicted by the person of ordinary skill that an IL-6 receptor antagonist would be as successful as it is in treating pancreatitis. Thus Applicants

discovery that administering an IL-6 antagonist can effectively prevent or ameliorate pancreatitis is a surprising and unexpected result.

D. Summary

The present claims are unobvious because the subject matter as a whole teaches away from the present invention. Even if the elements are found in the art, the art does not provide the requisite reason to combine them in the manner claimed, with predictable results. And, even if a *prima facie* case could be made, it is rebutted by teaching away and unexpected results. For all these reasons, the claims are nonobvious. Reconsideration and withdrawal are respectfully sought.

CONCLUSION

Applicants believe that the present application is in condition for allowance. Favorable reconsideration of the application is respectfully requested. The Examiner is invited to contact the undersigned by telephone if it is felt that a telephone interview would advance the prosecution of the present application.

The Commissioner is hereby authorized to charge any additional fees which may be required under 37 C.F.R. §§ 1.16-1.17, or credit any overpayment, to Deposit Account No. 19-0741. Should no proper payment be enclosed, the Commissioner is authorized to charge the unpaid amount to the Deposit Account. If any extensions of time are needed for timely acceptance of papers submitted herewith, Applicants petition under 37 C.F.R. §1.136 for such extensions and authorize payment of fees from the Deposit Account.

Respectfully submitted,

Date May 13, 2009 By /SElliott/

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APPENDIX A

Interleukin 6

From Wikipedia, the free encyclopedia

edit (http://en.wikipedia.org/w/index.php?title=Template:PBB/3569&action=edit)

Interleukin-6 (IL-6) is an interleukin that acts as both a pro-inflammatory and anti-inflammatory cytokine. It is secreted by T cells and macrophages to stimulate immune response to trauma, especially burns or other tissue

Interleukin 6 (interferon, beta 2)

Available structures: 1alu (http://www.rcsb.org/pdb/cgi/explore.cgi?pdbId=1alu), 1il6 (http://
(http://www.rcsb.org/pdb/cgi/explore.cgi?pdbId=1p9m), 2il6

Identifiers

Symbols	IL6 (http://www.genenames.org/data/hgnc_data.php?hgnc_id=6018); HGF	
External IDs	(OMIM 147620 (http://www.ncbi.nlm.nih.gov/entrez/dispomim.cgi?id
(http://www.informatics.jax.org/searches/access.cgi?report.cgi?id=MGI:	
Uniprot	P05231 (http://www.uniprot.org/uniprot/P05231)	
Refseq	NM_000600 (http://www.ncbi.nlm.nih.gov/entrez/viewer.fcgi?val=NM_000600) (mRNA) NP_000591 (http://www.ncbi.nlm.nih.gov/entrez/viewer.fcgi?val=NP_000591) (protein)	NM_031168 (http://ww
val=NM_031168) (mRN
NP_112445 (http://ww
val=NP_112445) (protei
Location	Chr 7: 22.73 - 22.74 Mb (http://genome.ucsc.edu/cgi-bin/hgTracks?org=Human&position=chr7:22732028-22738091)	Chr 5: 30.34 - 30.35 Mb
rg=Mouse&position=c
Pubmed search	[1] (http://www.ncbi.nlm.nih.gov/sites/entrez?db=gene&cmd=Link&LinkName=gene_pubmed&from_uid=3569)	[2] (http://www.ncbi.nl
b=gene&cmd=Link&I

Gene ontology

RNA expression pattern

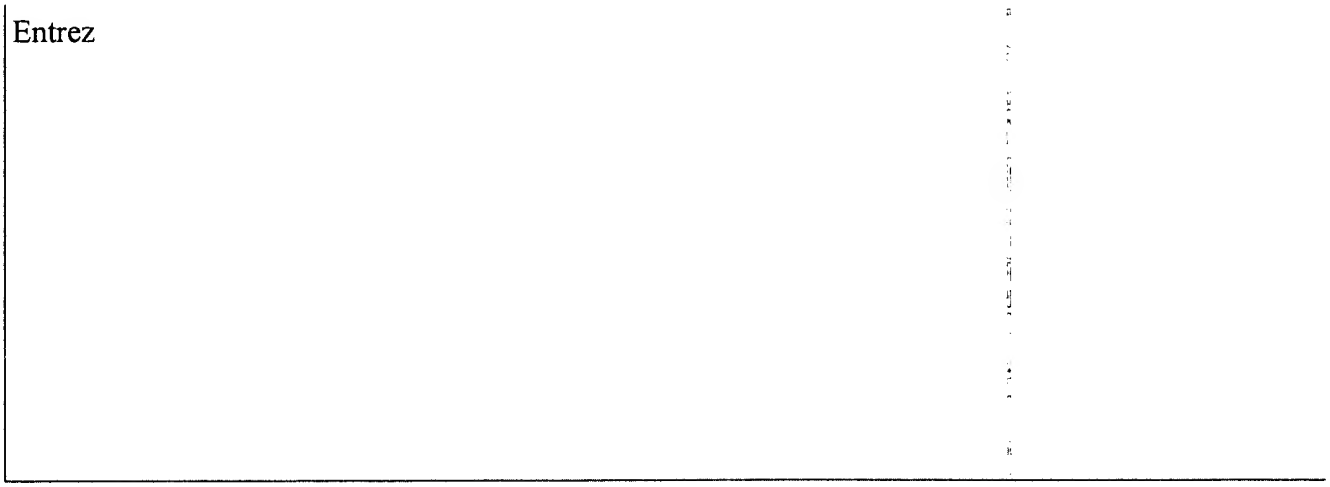
205207_at

GeneAtlas Tissues

More reference expression data (http://biogps.gnf.org/?query=HGI

Orthologs

Human	Mouse
3569 (http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?	16193 (http://www.ncbi



damage leading to inflammation. In terms of host response to a foreign pathogen, IL-6 has been shown, in mice, to be required for resistance against the bacterium, *Streptococcus pneumoniae*[1]. IL-6 is also a "myokine," a cytokine produced from muscle, and is elevated in response to muscle contraction[2]. It is significantly elevated with exercise, and precedes the appearance of other cytokines in the circulation. During exercise, it is thought to act in a hormone-like manner to mobilize extracellular substrates and/or augment substrate delivery (Petersen, J Appl Physiol 2005). Additionally, osteoblasts secrete IL-6 to stimulate osteoclast formation. Smooth muscle cells in the tunica media of many blood vessels also produce IL-6 as a pro-inflammatory cytokine. IL-6's role as an anti-inflammatory cytokine is mediated through its inhibitory effects on TNF-alpha and IL-1, and activation of IL-1ra and IL-10.

Contents

Functions of IL-6

IL-6 is one of the most important mediators of fever and of the acute phase response. In the muscle and fatty tissue IL-6 stimulates energy mobilization which leads to increased body temperature. IL-6 can be secreted by macrophages in response to specific microbial molecules, referred to as pathogen associated molecular patterns (PAMPs). These PAMPs bind to highly important group of detection molecules of the innate immune system, called pattern recognition receptors (PRRs), including Toll-like receptors (TLRs). These are present on the cell surface and intracellular compartments and induce intracellular signaling cascades that give rise to inflammatory cytokine production. IL-6 is also essential for hybridoma growth and is found in many supplemental cloning media such as briclone. Inhibitors of IL-6 (including estrogen) are used to treat postmenopausal osteoporosis. Il-6 is also produced by adipocytes and is thought to be a reason why obese individuals have higher endogeneous levels of CRP.

The IL-6 receptor

IL-6 signals through a cell-surface type I cytokine receptor complex consisting of the ligand-binding IL-

6Rα chain (CD126), and the signal-transducing component gp130 (also called CD130). CD130 is the common signal transducer for several cytokines including leukemia inhibitory factor(LIF), ciliary neurotropic factor, oncostatin M, IL-11 and cardiotrophin-1, and is almost ubiquitously expressed in most tissues. In contrast, the expression of CD126 is restricted to certain tissues. As IL-6 interacts with its receptor, it triggers the gp130 and IL-6R proteins to form a complex, thus activating the receptor. These complexes bring together the intracellular regions of gp130 to initiate a signal transduction cascade through certain transcription factors, Janus kinases (JAKs) and Signal Transducers and Activators of Transcription (STATs)[3].

IL-6 is probably the best studied of the cytokines that use gp130 in their signalling complexes. Other cytokines that signal through receptors containing gp130 are Interleukin 11 (IL-11), Interleukin 27 (IL-27), ciliary neurotrophic factor (CNTF), cardiotrophin-1 (CT-1), cardiotrophin-like cytokine (CLC), leukemia inhibitory factor (LIF), oncostatin M (OSM), Kaposi's sarcoma associated herpes virus interleukin 6 like protein (KSHV-IL6).[4] These cytokines are commonly referred to as the *IL-6 like* or *gp130 utilising* cytokines [5].

In addition to the membrane-bound receptor, a soluble form of IL-6R (sIL-6R) has been purified from human serum and urine. Many neuronal cells are unresponsive to stimulation by IL-6 alone, but differentiation and survival of neuronal cells can be mediated through the action of sIL-6R. The sIL-6R/IL-6 complex can stimulate neurites outgrowth promote survival of neurons, hence may be important in nerve regeneration through remyelination.

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APPENDIX B

Negative feedback

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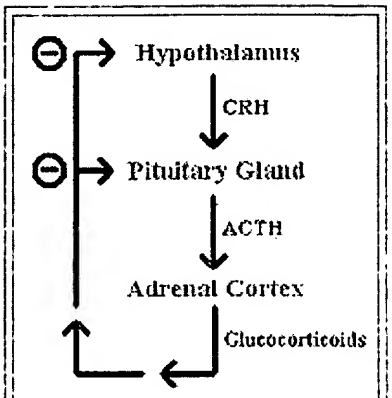
Negative feedback occurs when the output of a system acts to oppose changes to the input of the system; with the result that the changes are attenuated. All dynamical systems can be considered as a set of feedback loops. If the overall feedback of the system is negative, then the system will tend to be stable.



Most endocrine hormones are controlled by a physiologic **negative feedback** inhibition loop, such as the glucocorticoids secreted by the adrenal cortex. The hypothalamus secretes corticotropin-releasing hormone (CRH), which directs the anterior pituitary gland to secrete adrenocortocotropic hormone (ACTH). In turn, ACTH directs the adrenal cortex to secrete glucocorticoids, such as cortisol. As a check on the system, glucocorticoids not only perform their respective functions throughout the body but also negatively affect the release of further stimulating secretions of both the hypothalamus and the pituitary gland, effectively reducing the output of glucocorticoids once a sufficient amount has been released.[1]

Contents

Overview

In many physical and biological systems, qualitatively different influences can oppose each other. For example, in biochemistry, one set of chemicals drives the system in a given direction, whereas another set of chemicals drives it in an opposing direction. If one, or both of these opposing influences are non-linear, equilibrium point(s) result.

In biology, this process (generally biochemical) is often referred to as homeostasis; whereas in mechanics, the more common term is equilibrium.

In engineering, mathematics and the physical and biological sciences, common terms for the points around which the system gravitates include: attractors, stable states, eigenstates/eigenfunctions, equilibrium points, and setpoints.

Negative refers to the sign of the multiplier in mathematical models for feedback. In delta notation, -Δoutput is added to or mixed into the input. In multivariate systems, vectors help to illustrate how several influences can both partially complement and partially oppose each other.

In contrast, **positive feedback** is a feedback in which the system responds in the same direction as the perturbation, resulting in amplification of the original signal instead of stabilizing the signal. A positive feedback of 100% or greater will result in a runaway situation. Both positive and negative feedback

require a feedback loop to operate, as opposed to feedforward, which does not rely on a feedback loop for its control of the system.

Examples

Mechanical Engineering

Negative feedback was first implemented in the 16th Century with the invention of the centrifugal governor. Its operation is most easily seen in its use by James Watt to control the speed of his steam engine. Two heavy balls on an upright frame rotate at the same speed as the engine. As their speed increases they move outwards due to the centrifugal force. This causes them to lift a mechanism which closes the steam inlet valve and the engine slows. When the speed of the engine falls too far, the balls will move in the opposite direction and open the steam valve.

Control

Examples of the use of negative feedback to control its system are: thermostat control, phase-locked loop, hormonal regulation (see diagram above), and temperature regulation in animals.

A simple and practical example is a thermostat. When the temperature in a heated room reaches a certain upper limit the room heating is switched off so that the temperature begins to fall. When the temperature drops to a lower limit, the heating is switched on again. Provided the limits are close to each other, a steady room temperature is maintained. The same applies to a cooling system, such as an air conditioner, a refrigerator, or a freezer.

Biology

Some biological systems exhibit negative feedback such as the baroreflex in blood pressure regulation and erythropoiesis. Many biological process (e.g., in the human anatomy) use negative feedback. Examples of this are numerous, from the regulating of body temperature, to the regulating of blood glucose levels. The disruption of negative feedback can lead to undesirable results: in the case of blood glucose levels, if negative feedback fails, the glucose levels in the blood may begin to rise dramatically, thus resulting in diabetes.

For hormone secretion regulated by the negative feedback loop: when gland X releases hormone X, this stimulates target cells to release hormone Y. When there is an excess of hormne Y, gland X "senses" this and inhibits its release of hormone X.

Economics

In economics, automatic stabilisers are government programs which work as negative feedback to dampen fluctuations in real GDP.

Electronic amplifiers

The negative feedback amplifier was invented by Harold Stephen Black at Bell Laboratories in 1927, and patented by him in 1934. Fundamentally, all electronic devices (e.g. vacuum tubes, bipolar

transistors, MOS transistors) exhibit some nonlinear behavior. Negative feedback corrects this by trading unused gain for higher linearity (lower distortion). An amplifier with too large of an open-loop gain, possibly in a specific frequency range, will additionally produce too large of a feedback signal in that same range. This feedback signal, when subtracted from the original input, will act to reduce the original input, also by "too large" an amount. This "too small" input will be amplified again by the "too large" open-loop gain, creating a signal that is "just right". The net result is a flattening of the amplifier's gain over all frequencies (desensitising). Thus, though much more accurate, amplifiers with negative feedback can become unstable if not designed correctly, causing them to oscillate. Harry Nyquist of Bell Laboratories managed to work out a theory regarding how to make it stable.

Negative feedback is used in this way in many types of amplification systems to *stabilize* and improve their operating characteristics (see e.g., operational amplifiers).

See also

- Asymptotic gain model
- Biofeedback
- Control theory
- Cybernetics
- Harold Stephen Black
- Nyquist stability criterion
- Positive feedback
- Stability criterion
- Step response

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External links

- http://www.biology-online.org/4/1_physiological_homeostasis.htm

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APPENDIX C

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IL-6 INHIBITS LIPOPOLYSACCHARIDE-INDUCED TUMOR NECROSIS FACTOR PRODUCTION IN CULTURED HUMAN MONOCYTES, U937 CELLS, AND IN MICE[1]

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Incubation of the human U937 histiocytic lymphoma cell line with granulocyte-macrophage colony stimulating factor (GM-CSF) rendered the cells responsive to induction of TNF by LPS. Treatment with IL-6 reduced TNF production in GM-CSF-primed U937 cells. The inhibitory effect was most pronounced (≈80%) when IL-6 was added either along with GM-CSF or within the first 3 h of GM-CSF treatment. Both GM-CSF or IL-6 inhibited [^{3}H]TdR uptake in U937 cells, and simultaneous treatment with GM-CSF and IL-6 resulted in an additive inhibitory effect on cell proliferation. However, the inhibition of TNF production could not be explained by the inhibitory effect of IL-6 on cell growth, nor was it due to a reduction in cell viability. An inhibition of TNF production by IL-6 was also demonstrated in cultured human peripheral blood monocytes. Treatment with IL-6 also resulted in a dose-dependent reduction of the 17-kDa TNF band revealed by SDS-PAGE after labeling monocytes with [^{35}S]cysteine and immunoprecipitation with anti-TNF mAb. In addition, treatment with IL-6 resulted in a reduction of monocyte in vitro cytotoxicity for tumor target cells. Finally, in mice sensitized by the administration of Bacillus Calmette-Guérin, the injection of IL-6 significantly reduced the levels of TNF found in the serum upon challenge with LPS. Inasmuch as TNF is known to be an inducer of IL-6, the inhibitory action of IL-6 on TNF production may represent the negative arm of a regulatory circuit. The inhibitory action of IL-6 on TNF production is consistent with a predominantly antiinflammatory role of IL-6 in the intact organism.

IL-6,—also known as B cell stimulatory factor 2 (1), IFN-β_2 (2), 26-kDa protein (3), hybridoma/plasmacytoma growth factor (4), and hepatocyte stimulating factor (5)—is a multifunctional cytokine whose demonstrated actions include induction of terminal differentiation in B cells (6), stimulation of proliferation in T cells and thymocytes (7), induction of acute phase protein synthesis by hepatocytes (5), and stimulation of proliferation of various hematopoietic stem cells (8). The production of IL-6 is positively regulated in fibroblasts, monocytes, and other cells by a variety of stimuli including IL-1 (9), LPS, viruses, IFN-β, and platelet-derived growth factor (10). TNF too was shown to act as a potent stimulus for IL-6 production in fibroblasts and other cells (11, 12).

Many different types of cells have receptors for IL-6, including the histiocytic lymphoma cell line U937 (13). One of the original aims of the our study, was to determine if the spectrum of the biologic actions of IL-6 extends also to cells of the myelomonocytic lineage. It was recently shown that IL-6 indeed promotes the differentiation and maturation of normal myeloid precursor cells and myeloid leukemic cell lines (14, 15). In this study we examined the effect of IL-6 on the production of TNF in the human U937 cell line, induced to differentiate by the addition of GM-CSF[4] (16). We also examined the effect of IL-6 on the ability of human peripheral blood monocytes to produce TNF upon stimulation with LPS. In both cell systems treatment with IL-6 inhibited TNF production. Furthermore, IL-6 showed an inhibitory effect on TNF-mediated monocyte cytotoxicity to tumor target cells. The inhibitory effect of IL-6 on LPS-induced TNF production was also seen in mice sensitized by injection with BCG. Inasmuch as TNF is a potent inducer of IL-6, the inhibition of TNF production by IL-6 can be viewed as the negative arm of a regulatory circuit. Together with the stimulatory action on acute phase protein synthesis, the inhibitory effect of IL-6 on TNF production probably reflects a predominantly anti-inflammatory function of IL-6.

MATERIALS AND METHODS

Materials. Purified recombinant *Escherichia coli*-derived human TNF (sp. act. 4.5×10^7 U/mg) was generously provided by Dr. M. Tsujimoto of the Suntory Institute for Biomedical Research, Osaka, Japan. Purified recombinant human GM-CSF (sp. act. 4×10^7 U/mg) was kindly supplied by Dr. Steven Gillis (Immunex, Seattle, WA). The recombinant *E. coli*-derived human IL-6 (a kind gift from Drs. Toshio Hirano and Tadamitsu Kishimoto, Osaka University, Japan) had a sp. act. of 5×10^6 U/mg.

Cell lines. The A673/6 line is a subclone developed in our laboratory (17) from the rhabdomyosarcoma cell line A673 (originally supplied by Dr. George Todaro, National Cancer Institute, Bethesda, MD). The A673/6 line, found to be highly sensitive to the cytotoxic action of TNF, was maintained in Dulbecco's modified Eagle's medium (GIBCO, Grand Island, NY) with 10% FBS. The U937 histiocytic lymphoma line (provided by Drs. Rena Feinman and Jeffrey Ravetch, Memorial Sloan-Kettering Cancer Center, New York, NY) was maintained in RPMI 1640 medium (GIBCO) with 10% FBS. Murine L929

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[1] This study was supported by National Institutes of Health Grants AI-12948, CA-47304, and Contract NO1-CM-67895 from the National Cancer Institute.
[2] A.D. is a visiting scientist from the Tel-Aviv Medical Center and the Weizmann Institute of Science, Rehovot, Israel.
[3] Author to whom correspondence and requests for reprints should be addressed.

[4] Abbreviations used in this paper: GM-CSF, granulocyte-macrophage CSF; α_1-PI, α_1-proteinase inhibitor; α_2-M, α_2-macroglobulin; BCG, Bacillus Calmette-Guérin; CRP, C-reactive protein; FBS, fetal bovine serum.

cells were grown in MEM (GIBCO) supplemented with 10% FBS.

Assay for TNF cytotoxicity. TNF levels were determined by a highly sensitive and rapid bioassay developed in our laboratory, whose lower detection limit is about 1 pg/ml TNF. A673/6 cells were seeded 16 h before the assay in 96-well plates at a density of 3×10^4 cells/well. Immediately before the addition of the dilutions of test samples, the cells were treated with 20 μg/ml cycloheximide (Sigma Chemical Co., St. Louis, MO). After the addition of the samples and of dilutions of a recombinant TNF preparation used as the laboratory standard, the plates were incubated at 40°C in 5% CO_2. Incubation with cycloheximide at the elevated temperature of 40°C accelerates the development of cytotoxicity and increases the sensitivity of the assay. After 5 to 8 h the supernatants were discarded, the cells washed once with Ca^{2+}- and Mg^{2+}-free PBS to remove dead cells and debris, and the remaining viable cells were fixed with 10% formalin for 15 min, and then stained with a solution of 0.05% naphthol blue black in 9% acetic acid and 0.1 M sodium acetate (17). After 30 min the stain was poured off, the cells were washed twice with distilled water, and the dye eluted by addition of 150 μl of 50 mM NaOH. Absorbance of the eluted dye was read at 630 nm on a Dynatech Minireader II. The mean OD ± SD of quadruplicate determinations was plotted on a standard curve obtained with serial dilutions of the TNF laboratory standard. The results are expressed as pg/ml of TNF based on the cytotoxic activity of the TNF standard.

TNF induction in U937 cells. U937 cells were seeded in 24-well plates at a concentration of 10^5 cells/well. GM-CSF (10 ng/ml) was added to cells in the presence or absence of various IL-6 concentrations. After 24 h (unless specified otherwise), the U937 cultures were stimulated with 1 μg/ml LPS (from *E. coli* 0127:B8, Sigma). Six h later, the supernatants were collected and centrifuged to eliminate inadvertently collected cells. The samples were kept frozen at −70°C until examination of TNF levels by the cytotoxicity assay.

[^{3}H]TdR uptake. U937 cells were seeded in 96-well plates at a concentration of 5×10^3 cells/well in the presence of GM-CSF (10 ng/ml), IL-6 (range of concentrations 0.31 to 50 ng/ml), or various combinations of GM-CSF and IL-6, for 48 h. The cells were labeled by adding to each well 1 μCi [^{3}H]TdR (New England Nuclear, Boston, MA) in 50 μl RPMI 1640 medium 8 h before their harvest with the aid of a Skatron cell harvester. Results are expressed as mean [^{3}H] TdR uptake ± SD of triplicate determinations.

Monocyte isolation and culture. Human PBMC were isolated from the "buffy coats" of donated blood (kindly provided by Dr. Berish Rubin, The New York Blood Center, New York, NY), by centrifugation on Isolymph gradient (Teva, Jerusalem, Israel), followed by the removal of residual platelets, as described by Fischer et al. (18). Briefly, mononuclear cells at a concentration of 5×10^6/ml (in RPMI 1640 medium supplemented with 10% autologous serum) were incubated in autologous serum-coated tissue culture plates for 1 h at 37°C. After removal of nonadherent cells by repeated rinsing with warm serum-free RPMI 1640 medium, the adherent cells were detached by 20-min incubation at room temperature in the presence of 0.25% EDTA in PBS, pH 7.4. This procedure yields a cell population consisting of over 90% monocytes, as determined by α-naphthyl-acetate-esterase staining (18). The separated monocytes were distributed in 24-well plates at a concentration of 5×10^5 cells/well in RPMI 1640 medium. Before addition to the cultures, IL-6 was passed through a Detoxigel column (Pierce Chemical Co., Rockford, IL) to remove any possible traces of contaminanting LPS. At various times thereafter, the cultures were stimulated with 1 μg/ml LPS and, unless otherwise specified, the supernatants were collected 6 h later, and frozen at −70°C until TNF assay.

Monocyte cytotoxicity assay. A photometric quantitation of monocyte-mediated tumor cell cytotoxicity was performed according to the procedure of Leu and Herriott (19). Different concentrations of monocytes in RPMI 1640 medium were added to 96-well plates without or with IL-6 (40 ng/ml) and incubated for 24 h. Thereafter, 10^4 target cells (either A673/6 or L929) were added. Plates were further incubated for 48 h at 37°C in 5% CO_2. At the conclusion of the experiment, medium was decanted, the cells were fixed with absolute methanol for 5 min and then stained with Giemsa. Subsequently, the cells were washed with double distilled water, and after dye elution the absorbance of each well was read at 630 nm on a Dynatech Minireader II. Results are expressed as means of OD (± SD). The percentage of cytotoxicity was calculated from the mean OD by the equation:

$$\% \text{ cytotoxicity} = \left(1 - \frac{\text{absorbance (monocytes + target)} - \text{absorbance monocytes}}{\text{absorbance target}}\right) \times 100$$

Labeling of cells with [^{35}S]cysteine and immunoprecipitation of TNF. Monocytes separated as described above, were cultured in 24-well plates at a cell density of 5×10^5/well in the presence of different concentrations of IL-6. After 24 h, the culture supernatants were removed, the cells washed twice with cysteine-free RPMI 1640 medium (Select-Amine Kit, GIBCO) and incubated further in 250 μl cysteine-free medium per well in the presence of 1 μg/ml LPS and 100 μCi/ml L-[^{35}S]cysteine (New England Nuclear) for another 6 h. The culture supernatants were then removed and frozen at −70°C.

For immunoprecipitation (20) the collected monocyte culture supernatants were thawed and centrifuged for 7 min at 10,000 rpm. Aliquots of each sample, adjusted to contain equal counts, were mixed with 10 μg of mouse anti-human TNF mAb (kindly provided by Dr. David Wallach, Weizmann Institute, Rehovot, Israel). The samples were incubated at room temperature for 1 h on an orbital mixer, after which 14 μg rabbit anti-mouse IgG antibody (Sigma) was added to each sample. After mixing for an additional 30 min, immune complexes were precipitated by the addition to each sample of 100 μl of a 10% suspension of *Staphylococcus aureus* cells (Pansorbin, Cal-Biochem, La Jolla, CA). After another 30 min, the *S. aureus* cells were overlaid on a cushion of sucrose buffer (1 M sucrose, 0.15 M NaCl, 0.5% Nonidet P-40, 1% polyoxyethylene 20 cetyl ether) and centrifuged at 10,000 rpm for 2 min. The pellet was resuspended and washed once with sucrose buffer, twice with a buffer containing 0.15 M NaCl, 0.5% Nonidet P-40, 1% polyoxyethylene 20 cetyl ether and 5 mg/ml of BSA, and twice with 25 mM Tris·HCl buffer, pH 7.4. After the washing, the proteins bound to *S. aureus* were eluted by boiling for 5 min in SDS sample buffer containing β-mercaptoethanol. The immunoprecipitated proteins were fractionated by SDS-PAGE in 14% acrylamide. After fixation, staining and destaining, the gels were treated with EN^{3}HANCE (NEN, Boston, MA), dried and exposed to a Kodak XAR-5 film (Eastman Kodak, Rochester, NY).

Animal experiments. C57BL/6 male mice at 3 mo of age (Jackson Laboratory, Bar Harbor, ME) were injected with BCG according to the protocol used by Carswell et al. (21). Two weeks after BCG injection, the mice were divided in two groups. The first group received i.p. injections with 10 μg IL-6 in 0.2 ml PBS, whereas the control group received PBS only. Four h later, all mice received i.p. injections with 10 μg LPS each. After 120 to 140 min, blood was obtained from each mouse, left to coagulate at room temperature for 1 h, and the separated serum was frozen at −70°C until assay. TNF levels in each sample were determined by bioassay, as described above. To ascertain that cytotoxicity seen in the bioassay was due to TNF, all samples of mouse sera were also assayed in the presence of anti-mouse-TNF antibodies (kindly provided by Dr. Edward A. Havell, Trudeau Institute, Saranac Lake, NY).

RESULTS

Inhibitory effects of IL-6 on TNF production in U937 cells. In the process of studying the actions of IL-6 on myelomonocytic cells, we examined the effect of IL-6 on the generation of TNF by U937 cells. This cell line does not produce TNF either constitutively or in response to LPS stimulation unless the cells are first induced to differentiate, either by PMA or by a more physiologic stimulus, such as GM-CSF (16).

Coincubation of GM-CSF-treated U937 cells with IL-6 significantly reduced the release of TNF upon subsequent stimulation with a wide range of LPS concentrations (Fig. 1). The inhibitory action of IL-6 on TNF production by U937 cells primed with GM-CSF was dose dependent, becoming evident at IL-6 concentrations of about 3 ng/ml and maximal at about 25 ng/ml (Fig. 2). In other experiments TNF levels were examined at different times after LPS stimulation; the degree of reduction of TNF levels by IL-6 remained relatively constant, indicating that IL-6 did not simply alter the kinetics of TNF release (data not shown). Removal of IL-6 before the addition of LPS did not abolish the inhibitory action (not shown). The inhibition was not due to a toxic effect, because IL-6 did not impair U937 cell viability as judged by both trypan blue dye exclusion and by phase contrast microscopy (not shown).

Interestingly, whereas IL-6 antagonized the priming

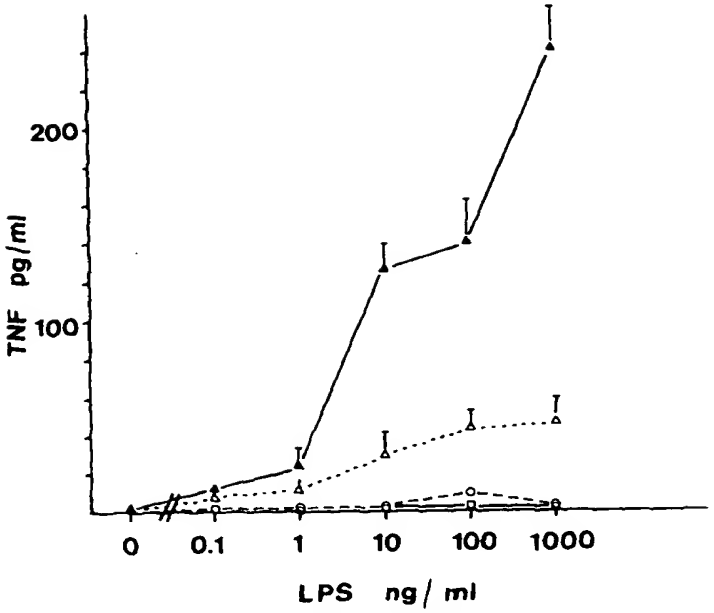

Figure 1. Inhibitory effect of IL-6 on TNF production in U937 cells. U937 cells (10^5/well) were incubated in 24-well plates with 10 ng/ml GM-CSF (▲), GM-CSF and 20 ng/ml IL-6 (△), IL-6 alone (○), or medium alone (□). After 24 h, the indicated concentrations of LPS were added to each well and the supernatants were collected 6 h later for TNF assay.

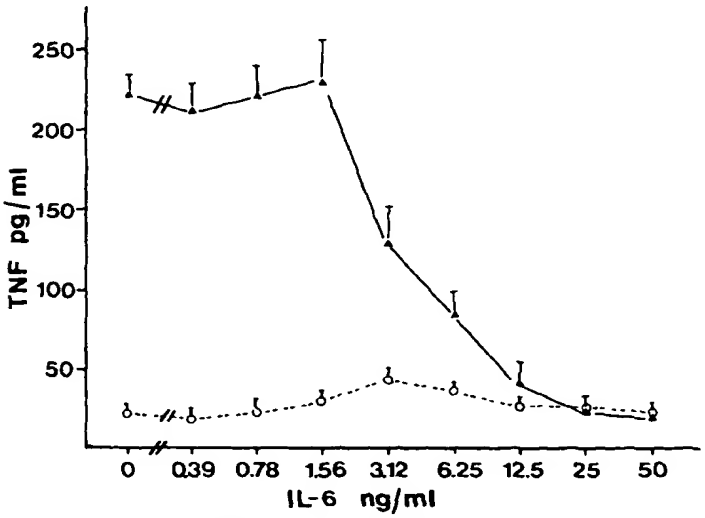

Figure 2. Effect of different concentrations of IL-6 on TNF production by U937 cells. U937 cells (10^5/well) were cultured for 24 h in 24-well plates with various concentrations of IL-6 in the presence (▲) or absence (○) of GM-CSF (10 ng/ml). All cultures were stimulated with 1 μg/ml of LPS 24 h after the initiation of the treatments. The supernatants were collected for TNF determination 6 h later.

effect of GM-CSF on TNF release, the two monokines acted in an additive manner in reducing [^{3}H]TdR uptake in the U937 cells (Fig. 3). Inhibition of colony growth or TdR uptake in U937 cells by GM-CSF (16) or IL-6 (22), respectively, were reported earlier. However, the inhibitory action of IL-6 on TNF production was not caused simply by a reduction in the cell number because IL-6 doses that lacked an inhibitory effect on proliferation significantly reduced TNF yields.

When IL-6 was added to U937 cells at different times after the onset of their treatment with GM-CSF, TNF production was affected in a stepwise fashion. The inhibitory effect was marked (≈80% reduction) if IL-6 was

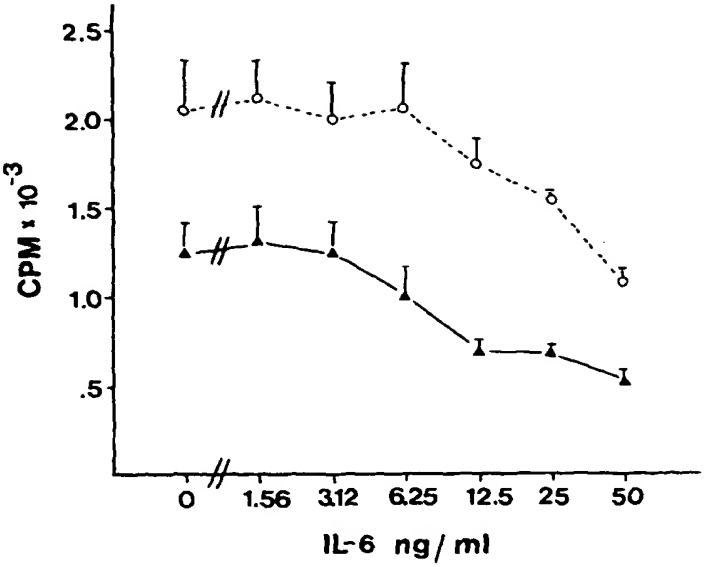

Figure 3. Inhibition of [^{3}H]TdR uptake in U937 cells by IL-6 and GM-CSF. U937 cells were treated for 48 h with increasing concentrations of IL-6 (○) or with the combination of GM-CSF (10 ng/ml) and various doses of IL-6 (▲). The results are expressed as mean [^{3}H]TdR uptake ± SD of triplicate examinations.

added up to 3 h after GM-CSF, but less prominent (≈50%) if IL-6 was added 4 to 24 h after the onset of GM-CSF treatment (Fig. 4). This biphasic response suggests that two different events might be involved in the inhibitory action of IL-6 on TNF release. It is noteworthy that an inhibitory effect was seen also when IL-6 was added 24 h after the onset of GM-CSF treatment, i.e., at the time of LPS addition.

Inhibitory effect of IL-6 on TNF production by peripheral blood monocytes. In subsequent experiments we addressed the question whether the observed inhibitory effect of IL-6 on TNF production can also be demonstrated in human peripheral blood monocytes. Our results demonstrate that IL-6 inhibited LPS-induced TNF production in cultured monocytes (Fig. 5). TNF yields were assayed in culture fluids collected at 6 h after LPS addition, i.e., at a time when TNF levels were found to reach a maximum under our experimental conditions. However, a similar degree of inhibition was also seen when TNF levels were examined at 18 or 24 h after LPS addition (data not shown). The inhibitory action of IL-6 was dose dependent, with some reduction in TNF yields seen with as little as 0.3 ng/ml of IL-6 and a 60 to 95% reduction reached at IL-6 concentrations of 20 to 40 ng/ml (Fig. 5). The inhibitory action was not diminished when, after a 12 to 24 h treatment with IL-6, the cells were washed and stimulated with LPS in the absence of IL-6. The degree of inhibition varied somewhat with cells from different donors. In general, the longer the preincubation of monocytes with IL-6, the more pronounced was the reduction in TNF release upon LPS stimulation. A 24-h incubation with IL-6 before LPS addition was more inhibitory than a 12-h incubation (Fig. 5 and data not shown). Simultaneous addition of IL-6 (40 ng/ml) and LPS to cultured monocytes also reduced TNF yields, measured 6 h after LPS addition, but only by about 45 to 50% (not shown).

Inhibitory effect of IL-6 on release of TNF quantitated by immunoprecipitation. Cytotoxicity measured in the

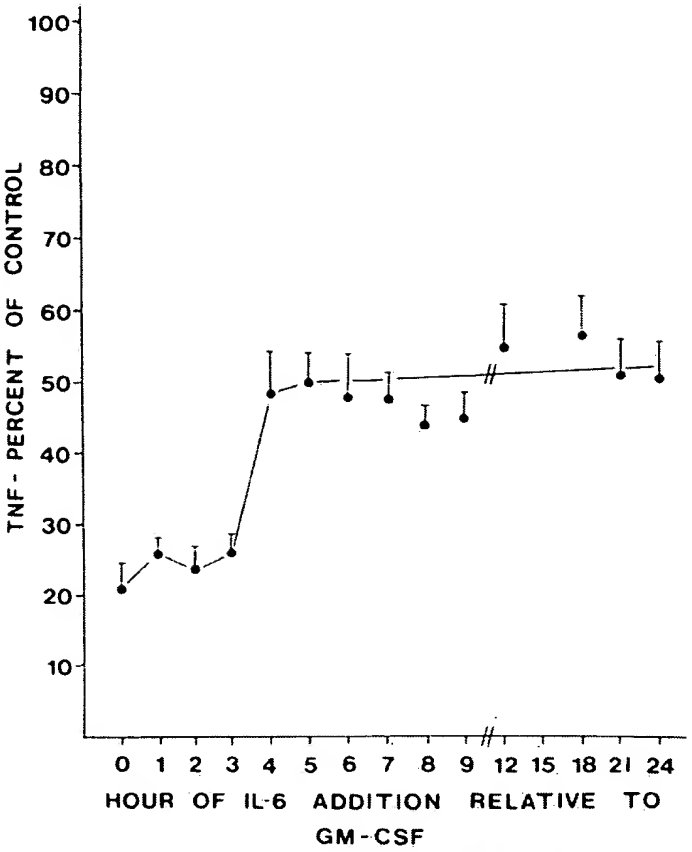

Figure 4. Inhibitory effect of IL-6, added at different times relative to GM-CSF, on TNF production by U937 cells. GM-CSF (10 ng/ml) was added to all cultures at time 0, whereas IL-6 (40 ng/ml) was added at various times thereafter. At 24 h all cultures were stimulated with 1 μg/ml LPS and 6 h later the supernatants were collected and examined for TNF content by assay in A673/6 cells as described in the *Materials and Methods*. Control GM-CSF-treated U937 cells stimulated with LPS yielded, in this experiment, 240 pg/ml of TNF (= 100%).

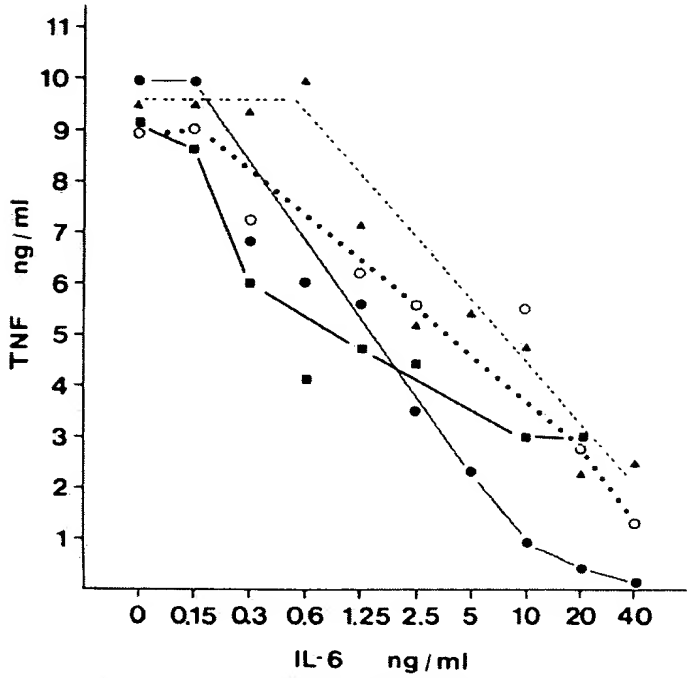

Figure 5. Effect of IL-6 on TNF production by peripheral blood monocytes. Monocytes (5×10^5/well) from four different donors were incubated in the presence of the indicated IL-6 concentrations for 12 h (○, ▲, ■) or 24 h (●) before LPS (1 μg/ml) addition. In cultures from two donors (●, ■) IL-6 was removed before LPS addition and incubation with LPS was conducted in the absence of IL-6. The supernatants were collected 6 h after the LPS addition and assayed for TNF in A673/6 cells.

TNF bioassay in A673/6 cells was due to TNF because the addition of a neutralizing murine mAb to human TNF completely neutralized the cytotoxic activity of supernatants from LPS-induced monocyte or U937 cell cultures (data not shown). Furthermore, the addition of IL-6 to TNF-containing samples before their assay in the A673/6 cells neither increased nor decreased the cytotoxic activity of TNF (not shown). Therefore, we are confident that the results shown in the earlier parts of this report are indeed due to an inhibitory action of IL-6 on the release of TNF, or at least the release of TNF in biologically active form. This conclusion is also borne out by the demonstration that treatment of peripheral blood monocyte cultures with IL-6 resulted in a dose-dependent inhibition of the release of [^{35}S]cysteine-labeled TNF, as visualized by SDS-PAGE after immunoprecipitation with a mAb specific for TNF (Fig. 6). The decrease in the 17-kDa band, corresponding to the released form of TNF (23), showed a good correlation with the IL-6 dose-dependent decrease in TNF activity measured in the bioassay (Fig. 5). Other experiments have shown that an unrelated mAb failed to immunoprecipitate, and that addition of an excess of unlabeled human rTNF competed with the ability of the anti-TNF antibody to immunoprecipitate, the 17-kDa [^{35}S]cysteine-labeled band, confirming that the 17-kDa band is indeed TNF (data not shown).

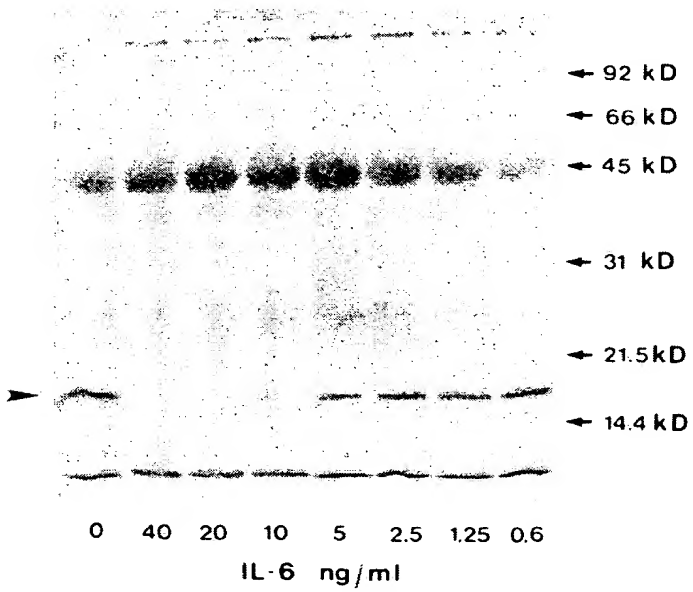

Figure 6. Immunoprecipitation of [^{35}S]cysteine-labeled TNF from monocyte cultures treated with different doses of IL-6. TNF produced in response to LPS by cultures of human peripheral blood monocytes, pretreated with different IL-6 concentrations for 24 h and labeled with [^{35}S] cysteine, was immunoprecipitated and fractionated by SDS-PAGE as described in *Materials and Methods*. The *arrow* on the *left* indicates the 17-kDa band of TNF. The samples loaded on the gel were from one of the experiments shown in Figure 5. The actual TNF yields, determined in the same samples by bioassay, are shown in Figure 5 (●); see points corresponding to the same IL-6 ng/ml in Figure 5 as marked under each lane in Figure 6. Positions of m.w. markers (Bio-Rad, Richmond, CA) are indicated on the *right*.

Inhibitory effect of IL-6 on TNF-mediated monocyte cytotoxicity. TNF is a major mediator of the tumoricidal effect of monocytes/macrophages (17, 24, 25). We addressed the question whether the effect of IL-6 on TNF production would be reflected in a reduced monocyte cytotoxicity for tumor cells. Indeed, IL-6 did impair monocyte-induced lysis of L929 cells (Fig. 7), as well as of A673/6 rhabdomyosarcoma cells (data not shown). Monocyte cytotoxicity for L929 and A673/6 cells is TNF dependent because the addition of the neutralizing mAb to TNF almost completely inhibited target cell lysis, both in the presence and absence of IL-6. Thus the decrease in monocyte cytotoxicity seen in the presence of IL-6 almost certainly can be ascribed to the inhibitory action of IL-6 on the generation of TNF.

Effects of IL-6 on TNF production in mice. To determine if the results obtained in cultures could be reproduced in intact animals, we examined the effect of IL-6 on LPS-stimulated TNF production in mice sensitized with BCG, as described in *Materials and Methods*. Preliminary experiments showed that TNF levels peaked at about 2 h after LPS administration. Therefore, in both TNF-treated and control mice, TNF levels in the blood were examined at 120 to 140 min after LPS injection (Fig. 8). All mice treated with IL-6 4 h before IL-6 injection had serum TNF concentrations below 70 pg/ml (mean ± SD = 42 ± 20 pg/ml) whereas all control mice had TNF concentrations more than 100 pg/ml (mean ± SD = 163.5 ± 58 pg/ml). This difference was statistically significant ($p < 0.01$ by Student's t-test).

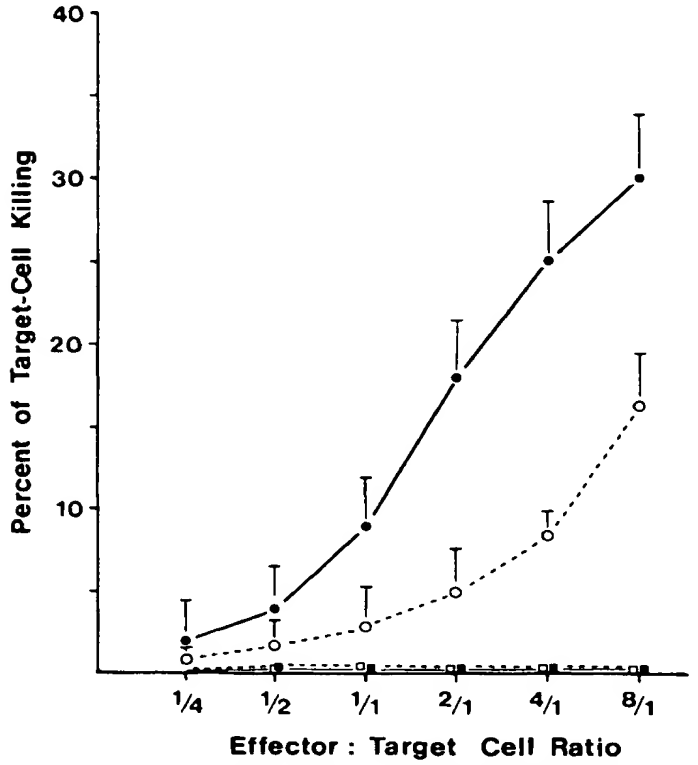

Figure 7. Inhibitory effect of IL-6 on monocyte cytotoxicity. Human peripheral blood monocytes were incubated for 24 h with control medium (●, ■) or 40 ng/ml IL-6 (○, □). Thereafter, murine L929 cells were added to the monocytes at different effector/target cell ratios. In addition, neutralizing mAb to TNF (final concentration 50 μg/ml) was added to another group of cultures treated either with medium (■) or 40 ng/ml IL-6 (□). Percent monocyte cytotoxicity was determined 48 h after the addition of the target cells as described in the *Materials and Methods*.

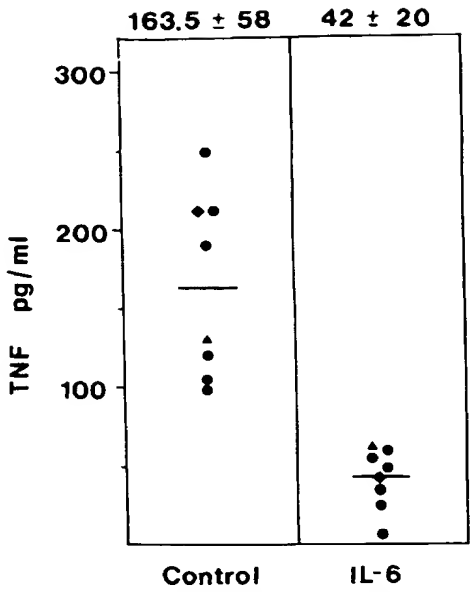

Figure 8. Effect of IL-6 on LPS-stimulated TNF production in mice. Mice sensitized with BCG were divided in two groups and injected i.p. with either PBS (control) or 10 μg IL-6 (see *Materials and Methods*). Four h later, each mouse was injected i.p. with 1 μg LPS. Blood was obtained from the mice at 120 min (●), 130 min (▲), or 140 min (◆) after LPS administration. Serum TNF levels were determined by cytotoxicity assay in A673/6 cells. *Horizontal lines* indicate mean TNF levels. Actual mean TNF levels (pg/ml ± SD) are given over each of the two panels.

To rule out that the inhibitory action of the IL-6 preparation is due to the presence of small quantities of LPS, we performed a separate experiment in which the IL-6 preparation was heated to 100°C to destroy all IL-6 activity without affecting LPS. The heated IL-6 preparation (10 μg/mouse) failed to cause any decrease in TNF levels whereas in the same experiments unheated IL-6 was highly effective (data not shown).

DISCUSSION

Our data illustrate a newly established link between TNF and IL-6, namely that IL-6 can reduce LPS-induced release of TNF in the U937 monocytoid cell line, in cultures of human peripheral blood monocytes, and in intact mice. This inhibitory action of IL-6 is of particular interest in view of the earlier demonstration that TNF is a potent inducer of IL-6 in cultured fibroblasts (11), in various tumor cell lines (12), and also in man (26). The existence of a reciprocal stimulatory/inhibitory interaction between TNF and IL-6 suggests a complex relationship of a considerable potential importance in the regulation of the many varied biologic actions of these two cytokines.

In U937 cells IL-6 effectively antagonized the priming effect of GM-CSF on TNF production. Yet, treatment of the U937 cells with IL-6 and GM-CSF resulted in an additive reduction in [^{3}H]TdR incorporation (Fig. 3). Reduction in cellular proliferation often accompanies cell differentiation. IL-6 was shown to promote differentiation in U937 cells (22) as well as in normal myeloid precursors or myeloid leukemic cells (14, 15). Thus the additive antiproliferative effects of IL-6 and GM-CSF may be related to the fact that both agents induce differentiation in U937 cells. It is possible that IL-6 induced differ-

entiation of U937 cells along a different maturation pathway than that promoted by GM-CSF, and that U937 cells committed to differentiate along the IL-6-mediated pathway are less competent to produce TNF in response to LPS. The time of IL-6 addition to U937 cells relative to GM-CSF affected the degree of inhibition of TNF production (Fig. 4). TNF release was markedly reduced (≈80%) if IL-6 was added up to 3 h after GM-CSF, but less affected (≈50%) when IL-6 was added at later times. This biphasic response suggests that two different events might be involved in the inhibitory action of IL-6 on TNF release from U937 cells. It is possible that IL-6 may affect an early process induced by GM-CSF, required to render the cells competent to release TNF in response to LPS. If added more than 3 h after GM-CSF, IL-6 may not be able to reverse this process, but it perhaps still reduces, to a lesser extent, the process of TNF synthesis or release per se.

Whereas in the U937 cells the inhibitory action of IL-6 might at least in part be due to an effect on cell differentiation, in the mature peripheral blood monocytes IL-6 is likely to have a more direct effect on some stage of TNF synthesis or release. The 17-kDa released form of TNF is derived from a 26-kDa intracellular precursor polypeptide (27). It has been postulated that the 26-kDa TNF form exists as an integral transmembrane protein (23), but the events regulating the processing and release of the 17-kDa form are not understood. The demonstration that IL-6 treatment of peripheral blood monocytes reduced the release of immunoprecipitable 17-kDa TNF after labeling with [^{35}S]cysteine (Fig. 6), suggests that actual synthesis of TNF is affected. Although unlikely, we cannot rule out the possibility that IL-6 affects the processing of the 26-kDa precursor, so that only the release of the active 17-kDa polypeptide, but not synthesis of the precursor form of TNF would be inhibited. The exact mechanism by which IL-6 modifies TNF production in peripheral blood monocytes, e.g., whether it inhibits TNF mRNA accumulation, is the subject of an ongoing study. To fully understand the events involved it will also be necessary to examine the effect of IL-6 on TNF receptor expression in monocytes or U937 cells, to ascertain whether increased utilization of TNF perhaps also might contribute to the observed decrease in TNF levels.

Inasmuch as TNF was shown to be an important mediator of tumor cell killing by monocytes (17, 24, 25), another question addressed was whether the inhibitory effect of IL-6 on TNF release is reflected in a reduced monocyte cytotoxicity for tumor cells. Indeed, IL-6 treatment did impair the lysis of L929 (Fig. 7) as well as A673/6 cells (data not shown) by peripheral blood monocytes. Monocyte cytotoxicity for both L929 and A673/6 cells is TNF dependent because the addition of a neutralizing mAb to human TNF almost completely inhibited target cell lysis, both in the presence or absence of IL-6 (Fig. 7 and data not shown). Thus the decrease in monocyte cytotoxicity for these cells seen in the presence of IL-6 almost certainly can be ascribed to the inhibitory action of IL-6 on the generation of TNF. Inasmuch as it was shown that monocyte cytotoxicity is likely to be mediated by the cell-associated (i.e., probably the 26-kDa) form of TNF, rather than by the released 17-kDa form (23, 28, 29), our results suggest that IL-6 inhibited the generation of 26-kDa TNF as well as released TNF.

It is interesting in this context that tumor cells have the capacity to generate several substances that can suppress the production of TNF and thereby may inhibit monocyte cytotoxicity. These substances include PGE2 (30), transforming growth factor-β (31) and, as we have shown, IL-6. The generation of these substances may enable tumor cells to evade TNF-mediated host defenses. However, IL-6 was found to promote differentiation of myeloid leukemia cells (14, 15) and to inhibit the growth of some carcinoma cell lines (22). Thus, it can be expected that depending on the type of neoplasm and specific conditions, endogenous or exogenous IL-6 might either promote or inhibit the development of malignancies.

IL-6 is a major inducer of the acute phase response, mediating the synthesis and release of CRP, α_2-M, α_1-PI, and other acute phase proteins (5). CRP is selectively deposited on necrotic cells that have phospholipids exposed on their damaged membranes (32). In addition, CRP may form complexes with cellular toxic constituents, such as chromatin fragments released into the circulation as a result of tissue damage, thereby perhaps facilitating their clearance (33). The function of α_1-PI and α_2-M is thought to be mainly antiinflammatory, as these proteins normally form a protective barrier between tissue and elastase—the latter being the most potent proteolytic enzyme released by activated neutrophils. TNF, released in septic conditions (34), may activate and prime neutrophils to release oxygen radicals (35). These radicals oxidize and irreversibly inactivate α_1-PI and α_2-M, thus exposing tissues to the digestive attack by the neutrophil elastase (36). By stimulating the acute phase response of the liver, IL-6 is triggering a process whose major function may be to indirectly counterbalance the toxic actions of TNF and other mediators of inflammation. Here we identified an additional potentially beneficial action of IL-6, i.e., its ability to reduce TNF production and thereby reduce the total inflammatory burden. Furthermore, because TNF cytotoxicity may be partly dependent on protease activity (37), IL-6-induced production of protease inhibitors by the liver may, theoretically, moderate TNF cytotoxicity.

Hepatocyte stimulating factor/IL-6 was also reported to induce ACTH release (38) and, consequently, cortisol—the best known antiinflammatory agent. It is interesting that some general similarities exist in the actions of IL-6 and glucocorticoids. Like IL-6, glucocorticoids are essential for the production of some acute phase reactants, especially α_2-M (39). The common repertoire of biologic actions of IL-6 and glucocorticoid hormones includes also the inhibition of TNF production (40).

TNF is similar in many of its actions to IL-1 (41), and both TNF and IL-1 are potent inducers of IL-6 (4, 11). In view of these similarities we thought that IL-6 might also inhibit IL-1 generation. However, in pilot experiments we failed to detect a significant effect of IL-6 on the release of IL-1β from LPS activated peripheral blood monocytes. If confirmed, this result would indicate that IL-6 does not affect the general process of monocyte activation by LPS but inhibits TNF production in a more selective manner. It is interesting that in addition to IL-6, TNF also stimulates the generation of GM-CSF (42, 43) which, in turn, can augment TNF release both in U937 cells and peripheral blood monocytes (16). Thus, TNF may potentially regulate its own production by generating both positive

(GM-CSF) and negative (IL-6) regulatory signals.

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APPENDIX D

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Rapid Communication

Intratracheal Injection of Endotoxin and Cytokines

II. Interleukin-6 and Transforming Growth Factor Beta Inhibit Acute Inflammation

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The nature of the endogenous mediators that downregulate and curtail the exodus of neutrophils into local acute inflammatory sites is unknown. In the present report, interleukin-6 (IL-6) and transforming growth factor beta (TGFβ), members of a family of macrophage-derived proteins known as cytokines, are shown to inhibit significantly the acute neutrophilic exodus caused by an intratracheal injection of endotoxin (LPS), a proinflammatory component of the cell walls of gram-negative bacteria. Transforming growth factor beta (10 μg) and IL-6 (10 μg) coinjected intratracheally with LPS (10 μg) each inhibited the number of neutrophils in 6-hour bronchoalveolar lavage (BAL) specimens by approximately 50%. The intratracheal coinjection of IL-6, TGFβ, and LPS inhibited the LPS-induced neutrophilic inflammatory exodus by nearly 75%. Interleukin-6 also is shown to be endogenously upregulated within the lung after intratracheal challenge with endotoxin, providing evidence that IL-6 may represent an endogenous negative feedback mechanism to inhibit endotoxin-initiated cytokine-mediated acute inflammation. Interleukin-6 and TGFβ both strongly inhibited the quantity of TNF-α recovered in the BAL fluid of LPS-challenged rats, suggesting that downregulation of LPS-induced TNF-α production within the lung represents one mechanism whereby IL-6 and TGFβ exert an antiinflammatory action. Interleukin-6 and TGFβ represent novel pharmacologic and, probably, endogenous inhibitors of acute inflammation. (Am J Pathol 1991, 138:1097–1101)

Interleukin-6 (IL-6) and transforming growth factor beta (TGFβ) are multifunctional members of a family of proteins, so called cytokines, that include the proinflammatory factors interleukin-1 (IL-1) and tumor necrosis factor (TNF). Cytokines are released by inflammatory and parenchymal cells in response to endotoxin (LPS), a proinflammatory lipopolysaccharide component of the cell walls of gram-negative bacteria. Cytokines are thought to orchestrate endotoxin-initiated acute inflammation (ie, the migration of neutrophils from blood vessels into tissues). Cytokines also contribute to many of the systemic effects of endotoxemia, such as neutrophilia, lymphopenia, fever, the synthesis of acute-phase proteins by the liver, and hypotensive shock.[1–3]

Intratracheal injection of endotoxin upregulates IL-1 and TNF expression in the lung and results in severe local acute inflammation.[4] Intratracheal injection of either IL-1 or TNF also induces acute inflammation in the lung.[4] Interleukin-1 is a far more potent inducer of acute inflammation than TNF in the lung as well as in other anatomic locations.[4–6]

The role of IL-6 in local acute inflammation has not, to our knowledge, been defined. Intravenous IL-6, however, was reported by Aderka et al[7] to decrease intravenous endotoxin-induced serum TNF levels in mice. Our laboratory has confirmed that intravenous IL-6 decreases intravenous endotoxin-induced serum TNF levels in rats.[8]

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In addition, and perhaps more importantly in regard to the pathogenesis of acute inflammation, we have observed that pretreatment with IL-6 decreases IL-1 mRNA levels in splenic and hepatic whole-organ RNA extracts of endotoxemic rats.[8] Because IL-6 downregulates endotoxin-induced TNF and IL-1 expression, and because IL-1 and TNF are thought to be proximal mediators of endotoxin-induced inflammation, an experimental animal model was created to test the hypothesis that IL-6 would downregulate LPS-induced acute inflammation and to search for local LPS-induced IL-6 expression.

The role of TGFβ in acute inflammation also has not, to our knowledge, been defined. Transforming growth factor beta, similar to IL-6, has been reported to be present in a variety of cell types, including activated macrophages.[9] Transforming growth factor beta has multiple complex growth stimulatory and growth inhibitory effects on mesenchymal and epithelial cells.[10] It is also a potent immunosuppressive factor. Transforming growth factor beta is present in rheumatoid synovial fluids and can inhibit IL-1–induced lymphocyte proliferation.[9] It has been reported to suppress the production of TNFα by LPS-stimulated macrophages.[11] In light of the known immunosuppressive effects of TGFβ, its possible role as an inhibitor of acute neutrophilic inflammation was investigated. Finally, because both IL-6 and TGFβ were found to act as pharmacologic inhibitors of local LPS-induced acute inflammation, we tested the anti-inflammatory effect of combined IL-6 and TGFβ.

Materials and Methods

Rats (congenic male Lewis rats weighing approximately 225g and anesthesized with ether) were injected intratracheally with equal volumes (0.5 ml) of various doses and combinations of either endotoxin (*S. Typhus* lipopolysaccharide, Sigma Chemical Co., St. Louis, MO), recombinant human IL-6, or recombinant human TGFβ$_1$ (gifts of Dr. Lawrence Souza, Amgen, Inc., Thousand Oaks, CA). Six hours later, the rats were killed and bronchoalveolar lavage (BAL) *via* the instillation of a tracheal catheter was performed to enumerate the absolute number of neutrophils in the intra-alveolar inflammatory exudate. The 6-hour timepoint previously has been documented by our laboratory[4] to represent the peak of the neutrophilic inflammatory exodus. Experiments were performed by randomly dividing rats into experimental groups to be injected on the same day with either LPS or with LPS plus IL-6 or TGFβ. A typical day's experiment consisted of injecting three rats intratracheally with LPS alone and three rats intratracheally with LPS plus a cytokine. The absolute number of neutrophils in the BAL specimens of the rats from each experimental group is expressed as the mean plus or minus one standard error of the mean. The probability value was determined by the two-tailed *t*-test for unpaired data. Histologic examination of Bouin's-fixed paraffin-embedded sections of the lungs after BAL was performed to evaluate the accumulation of neutrophils within pulmonary parenchyma and the adequacy of BAL. Tumor necrosis factor protein determinations in BAL fluid were determined as previously described.[12] Whole-lung RNA extraction, Northern blotting, and hybridization for IL-6 mRNA were performed as previously described.[12,13]

Results

The LPS-induced acute inflammatory exodus was inhibited by an average of 61% in rats receiving 10 μg IL-6 plus 1 μg LPS ($n = 7$), as opposed to rats receiving 1 μg LPS alone ($n = 9$) ($p < 0.0001$, Table 1). The LPS-induced neutrophilic exodus was inhibited by an average of 53% in rats receiving 10 μg IL-6 plus 10 μg LPS ($n = 18$), as opposed to rats receiving 10 μg LPS alone ($n = 18$) ($p < 0.0001$, Table 1). Intratracheal injection of IL-6 alone caused the accumulation of a very small number of neutrophils (less than 10^6 neutrophils/BAL, $n = 2$). Intratracheal injection of saline causes no or at most a very slight accumulation of neutrophils in BAL fluid.[4,14]

After having demonstrated that IL-6 can act as a pharmacologic downregulator of LPS-induced local acute inflammation, we wondered if there was evidence that host

Table 1. *IL-6 and TGFβ Inhibit Intratracheal LPS-induced Acute Inflammation*

Intratracheal injection	n	Neutrophils × 10^{-6}	Inhibition (%)	*P* value
LPS (1 μg)	9	6.36 ± 0.52	61	0.0001
LPS (1 μg) + IL-6 (10 μg)	7	2.45 ± 0.54		
LPS (10 μg)	18	20.86 ± 0.17	53	0.0001
LPS (10 μg) + IL-6 (10 μg)	18	9.84 ± 0.12		
LPS (10 μg)	6	12.67 ± 1.10	35	0.02
LPS (10 μg) + TGFβ (1 μg)	6	8.22 ± 1.20		
LPS (10 μg)	14	18.74 ± 1.06	54	0.0001
LPS (10 μg) + TGFβ (10 μg)	13	8.55 ± 0.57		

LPS, Lipopolysaccharide, TGFβ, transforming growth factor beta.

IL-6 is upregulated locally after the intratracheal injection of LPS and may therefore act as an endogenous negative feedback mechanism to stop the neutrophilic exodus that is the cellular hallmark of acute inflammation. Interleukin-6 mRNA expression was determined in whole-lung RNA extracts by Northern blotting using a rat IL-6 cDNA probe[13] at sequential timepoints after the intratracheal or intravenous injections of 100 μg LPS. Intratracheal injection of LPS caused IL-6 mRNA expression beginning at 2 hours and increasing markedly at 4 hours (Figure 1). In a further study of pulmonary IL-6 mRNA expression at later timepoints after intratracheal injection of LPS, IL-6 mRNA expression was found to remain at a very high level at 6 hours with a return to preinjection levels at 12, 24, and 48 hours (data not shown). In contrast to the kinetics of IL-6 expression after the intratracheal injection of LPS, intravenous injection of LPS caused IL-6 mRNA expression to peak at 2 hours with a substantial decrease toward baseline level by 4 hours (Figure 1).

The LPS-induced acute inflammatory exodus was inhibited by an average of 54% in rats receiving 10 μg TGFβ plus 10 μg LPS ($n = 13$), as opposed to rats receiving 10 μg LPS alone ($n = 14$) ($p < 0.0001$, Table 1). The LPS-induced accumulation of neutrophils was inhibited by 35% in rats receiving 1 μg TGFβ plus 10 μg LPS ($n = 6$), as opposed to rats receiving 10 μg LPS alone ($n = 6$) ($p < 0.02$, Table 1). Intratracheal injection of TGFβ alone did not cause a significant influx of neutrophils into the lungs (less than 0.1×10^6 neutrophils/BAL).

The combined effects of IL-6 and TGFβ on LPS-induced acute neutrophilic inflammation then were investigated. All four experimental groups (10 μg LPS alone, 10 μg LPS plus 10 μg IL-6, 10 μg LPS plus 10 μg TGFβ, and 10 μg LPS plus 10 μg IL-6 plus 10 μg TGFβ) were studied concurrently. The combination of IL-6 plus TGFβ inhibited the LPS-induced neutrophilic exodus by 74%, as opposed to 35% inhibition with IL-6 alone and 56% inhibition with TGFβ alone (Table 2). The combined inhibitory effect of IL-6 and TGFβ was significantly greater than the inhibitory effect of either cytokine alone ($p < 0.008$).

Histologic examination of the lungs in all four experimental groups ($n = 2$ rats from each group) was performed after BAL to exclude the possibility that the decrease in BAL neutrophils might be due to an increase in neutrophils remaining in the lungs of cytokine-treated rats (as might occur, for example, if IL-6 or TGFβ induced the expression of adhesion molecules that caused the neutrophils to adhere more avidly to pulmonary parenchyma). Double-blind microscopic evaluation of the lungs revealed an indistinguishable histologic appearance in the LPS-plus-cytokine–treated groups. The LPS-alone–treated group was distinguished from the other three groups by a greater number of neutrophils. One may therefore conclude that the degree of cytokine-induced inhibition of acute neutrophilic inflammation as measured by BAL is, if anything, an underestimation of the true amount of inhibition. In none of the experimental groups (not even after intratracheal injection of 10 μg LPS

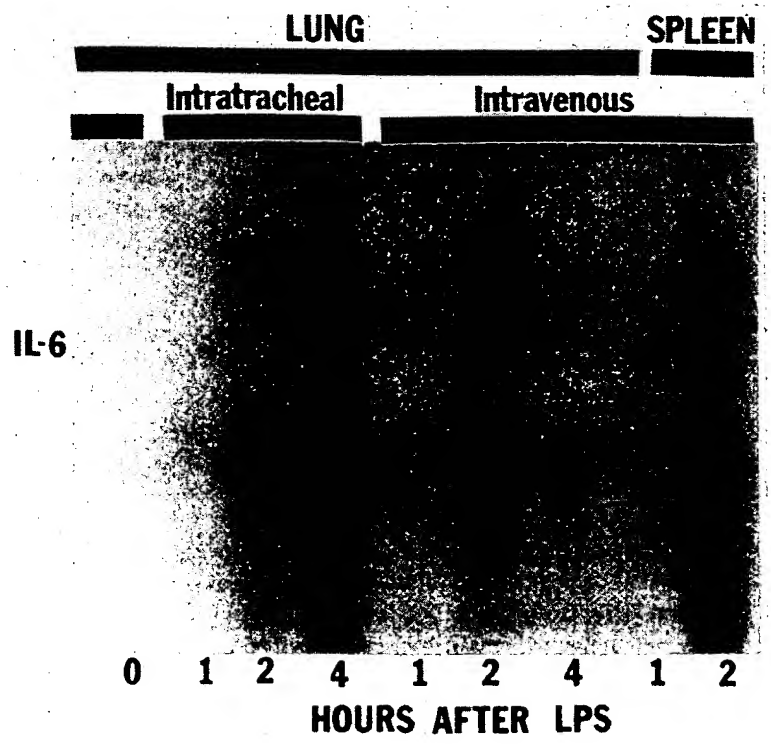

Figure 1. *IL-6 mRNA is not constitutively detected in whole-lung RNA preparations of naive rats (time 0) or 1 hour after either intratracheal or intravenous injections of endotoxin. IL-6 mRNA is detectable in the lung at similar levels at 2 hours after either the intratracheal or intravenous injections of endotoxin. At 4 hours after the intratracheal injection of endotoxin, the level of pulmonary IL-6 mRNA has increased dramatically, whereas at the same timepoint after the intravenous injection of endotoxin the level of IL-6 has almost returned to preinjection level. The 4-hour timepoint marks the beginning of the neutrophilic exodus into alveoli after the intratracheal, but not the intravenous, injection of endotoxin.[4] The expression of splenic IL-6 mRNA in a whole-organ RNA preparation at 2 hours after the intravenous injection of endotoxin is shown for comparison. All lanes of the Northern blot were loaded with 25 μg whole-organ RNA and hybridized with rat IL-6 cDNA. The rat expresses two major IL-6 mRNA species that code for the same protein.[13]*

Table 2. *The Combination of IL-6 and TGFβ Inhibits Intratracheal LPS-induced Acute Inflammation More Than Either Cytokine Alone*

Intratracheal injection	n	Neutrophils × 10^{-6}	Inhibition (%)	*P* value
LPS (10 μg)	8	20.75 ± 1.42	—	—
LPS (10 μg) + IL-6 (10 μg)	8	13.44 ± 1.39	35	0.002
LPS (10 μg) + TGFβ (10 μg)	7	9.10 ± 0.88	56	0.0001
LPS (10 μg) + IL-6 (10 μg) + TGFβ (10 μg)	8	5.50 ± 0.75	74	0.0001

LPS, lipopolysaccharide; TGFβ, transforming growth factor beta.

alone) was any evidence of architectural or cellular injury to the lung observed at the light microscopic level. The LPS-induced inflammatory reaction was histologically characterized by the appearance of a modest number of neutrophils in alveolar septae, alveolar spaces, and in bronchial mucosa, as previously illustrated by our laboratory.[4] Tumor necrosis factor protein levels in the BAL fluids of the same rats were measured and were found to be decreased by 66% in both LPS-plus-IL-6– and LPS-plus-TGFβ–treated rats and to be decreased by 88% in LPS-plus-IL-6-plus-TGFβ–treated rats as compared to LPS-alone–treated rats (274 ± 30 U TNF/BAL after LPS alone, 94 ± 36 U TNF/BAL after LPS plus IL-6, 96 ± 38 U TNF/BAL after LPS plus TGFβ, and 34 ± 17 U TNF/BAL after LPS plus IL-6 plus TGFβ).

Discussion

Although much new information has emerged during the past few years regarding the acute proinflammatory effects of such cytokines as IL-1 and TNF, relatively little is known about endogenous downregulators of acute inflammation. In the present study, IL-6 and TGFβ, alone and especially in combination, are shown to inhibit strongly LPS-induced acute inflammation. Interleukin-6 and TGFβ thus represent novel antiinflammatory agents of potential therapeutic utility in clinical situations of cytokine-mediated acute inflammation.

Neutrophils first appear in BAL fluids 4 hours after intratracheal injection of endotoxin,[4] whereas neutrophils are not present in BAL fluid 4 hours after the intravenous injection of endotoxin. The marked increase in pulmonary IL-6 mRNA after intratracheal as opposed to intravenous injection of endotoxin therefore may be, in part, neutrophil derived, especially because Cicco et al[15] recently reported that neutrophils express IL-6 in response to stimulation with endotoxin *in vitro*. The concept that neutrophils may downregulate their own accumulation *via* the expression of IL-6 is attractive, but our data do not allow us to quantitate the probable increased contribution of alveolar macrophage-derived IL-6 after intratracheal administration as opposed to intravenous administration of endotoxin. Endotoxin-stimulated cells of the monocyte–macrophage lineage are known to secrete large amounts of IL-6.[16] The amounts of IL-6 and TGFβ that are released into the alveolar fluid of the lung under pathophysiologic conditions are unknown. Interleukin-6 is known, however, to attain concentrations as high as 2 μg/ml in the serum of endotoxemic rats,[8] suggesting that microgram quantities of endogenous IL-6 also might be attainable in the lung during gram-negative pneumonia.

In conclusion, pharmacologic administration of exogenous recombinant IL-6 and TGFβ inhibits endotoxin-induced local acute inflammation. The mechanism of the anti-inflammatory action of IL-6 and TGFβ may relate to the ability of these cytokines to inhibit TNFα[8,11] (and as shown by the present data) and IL-1 production[8] by macrophages. Host-derived IL-6 is upregulated locally after challenge with LPS and may act as an endogenous-negative feedback mechanism to inhibit the LPS-initiated IL-1– and TNF-mediated acute inflammatory process. Finally host macrophage-derived TGFβ may be speculated also to play a role as an endogenous inhibitor of acute inflammation. Transforming growth factor beta has been reported by Roberts et al[17] to increase collagen synthesis *in vitro* and to cause fibrosis *in vivo* after intradermal injection. Transforming growth factor beta is known to be a pleiotropic mediator and may be hypothesized to perform a dual role by acting both to resolve acute inflammation and to begin tissue repair and remodeling by initiating collagen deposition.

Acknowledgments

The authors thank Dr. Lawrence Souza and Dr. Keith Wescott (Amgen, Inc., Thousand Oaks, CA) for providing the recombinant human IL-6 and TGFβ and Drs. W. Northemann and G. Fey (Scripps Research Foundation, La Jolla, CA) for providing rat IL-6 cDNA.

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APPENDIX E

Digestive Diseases and Sciences, Vol. 42, No. 8 (August 1997), pp. 1783–1788

Tissue-Specific Cytokine Production During Experimental Acute Pancreatitis

A Probable Mechanism for Distant Organ Dysfunction

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Our purpose was to determine if cytokines are produced systemically during acute pancreatitis. Proinflammatory cytokines are elevated during acute pancreatitis and have been implicated in the progression of pancreatitis-associated multiple organ dysfunction. Whether these mediators are produced within all tissues or very few specific organs is not known. Edematous pancreatitis was induced in adult male mice by IP injection of cerulein. Necrotizing pancreatitis was induced in young female mice by feeding a choline-deficient, ethionine supplemented diet. Animals were sacrificed as pancreatitis worsened, with multiple organs prepared for tissue mRNA and protein analysis by RT-PCR and immunoblotting. Pancreatitis severity was established by histologic grading and serum amylase and lipase. There was no cytokine mRNA or protein detectable prior to the induction of pancreatitis. Tumor necrosis factor-alpha (TNF-α) and interleukin-1-beta (IL-1β) mRNA and protein were detected within the pancreas early in the course of pancreatitis in both models, coinciding with the development of hyperamylasemia (both $P < 0.001$). Interleukin-6 was produced in the pancreas after pancreatitis was more fully developed ($P < 0.001$). IL-1β and TNF-α were subsequently produced in large amounts in lung, liver, and spleen but never within kidney, cardiac muscle, or skeletal muscle. A significant delay between pancreatic and distant organ cytokine production was always observed. It is concluded that proinflammatory cytokines are produced within the pancreas and within organs known to develop dysfunction during severe pancreatitis. Cytokine production is tissue specific, correlates with disease severity, and occurs within the pancreas first and subsequently within distant organs.

KEY WORDS: interleukin-1; interleukin-6; tumor necrosis factor; gene expression; acute pancreatitis.

Acute pancreatitis remains a serious multisystem pathologic condition with an unacceptably high morbidity and mortality (1, 2). Although the prognosis varies to some extent with the severity of pancreatic destruction, it is the extrapancreatic manifestations such as respiratory, cardiovascular, and hepatic dysfunction that contribute most significantly to the morbidity and mortality of this disease (2–4). The mechanism responsible for the involvement of these distant organ systems is unclear, but recent evidence suggests the proinflammatory cytokines interleukin-1-beta (IL-1β), interleukin-6 (IL-6), and tumor necrosis factor-alpha (TNF-α) may propagate end-organ dysfunction.

Clinical studies have shown IL-1β, IL-6, and

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TABLE 1. MURINE CYTOKINE cDNA PRIMERS

Cytokine	*Sense primer (5′ × 1°)*	*Antisense primer (5′ × 3′)*	*Product size (bp)*
IL-1β	CAGGATGAGGACATGAGCACC	CTCTGCAGACTCAAACTCCAC	447
TNF-α	ATGAGCACAGAAAGCATGATC	TACAGGCTTGTCACTCGAATT	276
IL-6	GACAAAGCCAGAGTCCTTCAGAGAG	CTAGGTTTGCCGAGTAGATCTC	229
β-Actin	GTGGGCCGCTCTAGGCACCA	CGGTTGGCCTTAGGGTTCAGGGGG	245

TNF-α in the serum of patients with acute pancreatitis and the degree of cytokine elevation correlating with disease severity and overall morbidity (5–8). A number of animal models have confirmed the role played by proinflammatory cytokines by demonstrating that serum concentrations correlate well with pancreatitis severity and are predictive of eventual mortality (9–13). When the IL-1β receptor is blocked during experimental pancreatitis, pancreatic as well as pulmonary inflammation and damage is attenuated, hypotension is prevented, and survival is greatly increased (14–16). Similarly, when circulating TNF-α is antagonized, experimental pancreatitis is less severe and the associated lung injury and mortality is decreased (17–20). More recent investigations utilizing transgenic knockout animals have demonstrated that active cytokine receptors are required for the development of maximal intrapancreatic and systemic (pulmonary) inflammation and destruction (21). These studies provide evidence that the development of systemic organ dysfunction typical of severe clinical pancreatitis may be cytokine mediated.

Although large amounts of IL-1β and TNF-α are known to be produced within the pancreas (9, 10, 20, 22), it is unclear if this is the sole source for the high levels found systemically or whether other remote sites are somehow recruited into cytokine production. We hypothesized that organs involved in the systemic manifestations of acute pancreatitis (ie, lung, liver) will have increased tissue levels of proinflammatory cytokines, while those organs not involved (ie, heart, skeletal muscle) will not have elevated tissue cytokine levels.

MATERIALS AND METHODS

Animal Model. Animal studies were performed at an AAALAC-accredited facility in accordance with the Department of Laboratory Animal Medicine at the University of South Florida. To avoid model-based bias, two well-described models of acute pancreatitis were used: (1) Acute edematous pancreatitis was induced in adult male C57/BL6 mice (33.2 ± 1.0 g) by intraperitoneal administration of the cholecystokinin agonist cerulein (Bachem, Torrence, California) (50 μg/kg every hour × 4) (23). Twenty animals were sacrificed at regular intervals as pancreatitis developed (0, 1, 2, 4, and 6 h). (2) Acute hemorrhagic, necrotizing pancreatitis was induced in young female NIH Swiss mice (14.8 ± 0.7 g) by the feeding of a choline-deficient, ethionine supplemented (CDE) diet (Harlan Teklad, Madison, Wisconsin) for 48 h (15). Twenty animals were sacrificed at 0, 12, 24, 48, and 72 h.

Tissue Preparation. Following pentobarbital anesthesia (50 mg/kg intraperitoneally), animals from each group were sacrificed by exsanguination via cardiocentesis. Intracardiac injection of 3 ml sterile PBS was utilized to purge the circulatory system and then the pancreas, lungs, liver, kidneys, and spleen and the cardiac and gastrocnemius muscles were immediately excised and divided for light microscopy (pancreata) and the isolation of protein and total RNA (all organs). The severity of pancreatitis was verified in all animals by serum amylase, lipase, and blind histologic grading as described previously (17, 24).

Measurement of Tissue Cytokine mRNA by Semiquantitative Differential Polymerase Chain Reaction. Total RNA was immediately isolated from each tissue by guanidium thiocyanate/acid phenol extraction as previously described (25). The integrity of isolated RNA was verified by equimolar 18S and 28S ribosomal RNA bands following denaturing electrophoresis. RNA was subsequently primed using oligo $(dt)_{12-15}$ (Gibco, Gaithersburg, Maryland) and then reverse transcribed (26). The prepared cDNA then underwent differential PCR with murine-specific primers for IL-1β, TNF-α, IL-6, and β-actin by coamplifying for 30 cycles in the presence of 5′ and 3′ primers specific for an individual cytokine and β-actin (Table 1). The reaction products were subjected to electrophoresis in 2.5% Metaphor agarose (FMC Bioproducts, Rockland, Maine) containing ethidium bromide and photographed digitally under ultraviolet light. Band intensity was determined by optical density with individual cytokine/β-actin cDNA ratios compared using Sigma Scan software (Jandel Scientific, San Rafael, California). All primers are known to span at least one intron.

The internal standard (β-actin) has previously been shown by our laboratory to be linear and unaffected during these experimental models while maintaining a linear relationship with IL-1β, TNF-α, and IL-6 for 20–40 amplification cycles (22, 26). All cDNA products were verified by restriction digestion (IL-1β via PstI, TNF-α via AvaII, and IL-6 via AluI), yielding the anticipated fragments of 340 and 103 bp for IL-1β, 156 and 120 bp for TNF-α, and 108 and 121 bp for IL-6.

Tissue Cytokine Protein. Protein was isolated from whole tissue homogenates (Tris HCl buffer, pH 7.4), standardized to serum protein concentration (7.5 mg/dl), and subsequently analyzed via western immunoblotting. Immunoblots were prepared following SDS-PAGE as outlined by

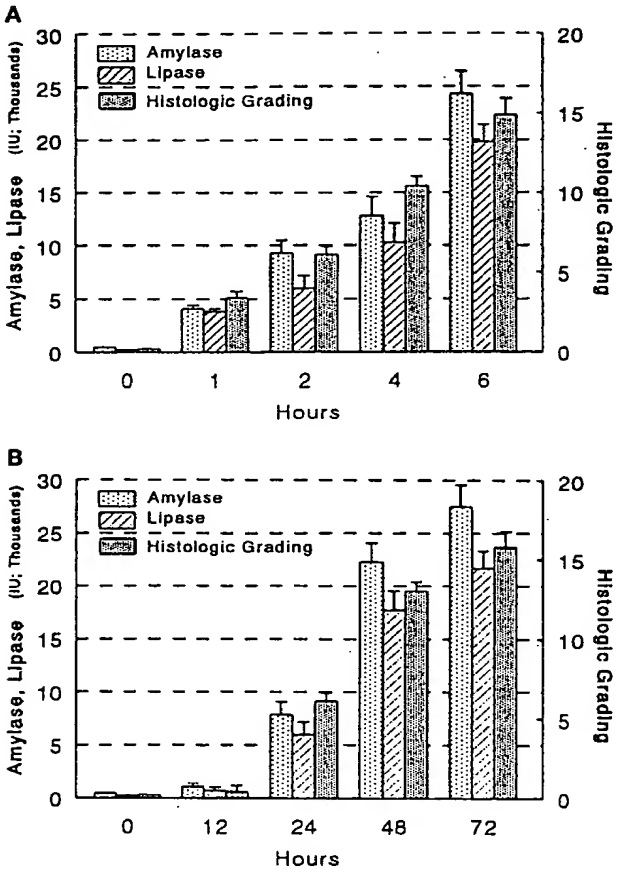

Fig 1. Assessment of pancreatitis severity. Serum amylase, lipase, and blind histologic grading are shown during the development of edematous (A) and necrotizing (B) pancreatitis. All three parameters $P < 0.001$ vs baseline.

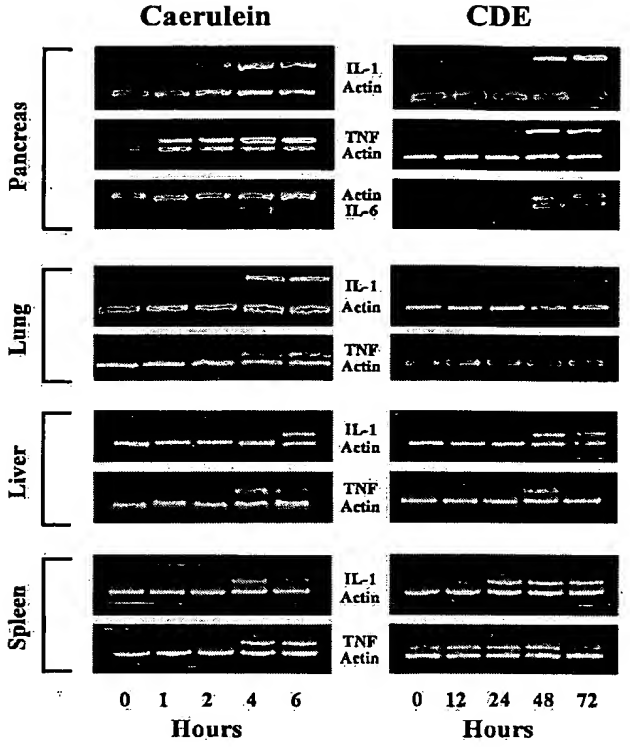

Fig 2. Systemic cytokine mRNA. The RT-PCR products of IL-1β, TNF-α, and IL-6 obtained from multiple tissues during the development of cerulein-induced edematous or CDE diet-induced necrotizing pancreatitis are shown. The internal standard (β-actin) was coamplified with each cytokine. There was no constitutive (baseline) expression of any cytokine mRNA. IL-1β and TNF-α mRNA were detected within the pancreas coincident with the development of histologic and biochemical evidence of pancreatitis, increasing with severity. Message within the other tissues was also significantly up-regulated, but at a later time point as pancreatitis worsened.

our laboratory previously (24) using polyclonal rat anti-mouse IL-1β, IL-6, and TNF-α antibodies (IL-1β and IL-6 from PharMingen, San Diego, California; TNF-α from Endogen, Boston, Massachusetts). Quantification of the resulting bands was by optical scanning as above.

Statistical Analysis. Results are expressed as means ± SEM. Statistical analysis was performed using the StatS 3 statistical program (Spreadware, Palm Desert, California) applying the unpaired two-tailed Student's t test with significance being assigned to P values <0.05 unless stated otherwise.

RESULTS

Development of Pancreatitis. Acute edematous pancreatitis was demonstrable 1 hr following the initial cerulein injection and progressively worsened over 6 hr to include partial necrosis as determined by histologic scoring and elevations in serum amylase and lipase (all three $P < 0.001$ vs baseline; Figure 1A). Hemorrhagic necrotizing pancreatitis developed over a period of two days in the animals fed CDE diet, with significant elevations in amylase, lipase, and histologic scores first detectable at the 24-h time point (all $P < 0.05$ vs baseline), reaching severe necrosis and inflammation at 72 h (Figure 1B).

Induction of Cytokine mRNA. There was no detectable mRNA for IL-1β, IL-6, or TNF-α within any tissue in normal (baseline) animals. Concomitant with the induction of pancreatitis, mRNA for all three proinflammatory cytokines was readily detected within the pancreatic parenchyma (Figure 2). The rise in these gene products was similar for both models of pancreatitis in that IL-1β and TNF-α became elevated when the first biochemical or histologic signs of pancreatitis became evident and peaked when these same parameters were at their maximum (both $P < 0.001$ vs baseline). IL-6 mRNA on the other hand,

was not up-regulated until the pancreatitis was more fully developed ($P < 0.005$ vs baseline).

During the progression of pancreatitis, IL-1β and TNF-α (but not IL-6) were dramatically induced in the lungs, spleen, and liver. The amount of cytokine mRNA in these organs increased as pancreatitis worsened and peaked coincident with maximum histologic pancreatitis ($P < 0.001$ vs baseline, Figure 2). Kidney, skeletal muscle, and cardiac muscle failed to demonstrate cytokine mRNA at any time point regardless of the pancreatitis model used (data not shown).

A significant amount of time expired after cytokine gene induction was apparent within the pancreas before the corresponding mRNA could be found in distant tissues. Again, these findings were not model-dependent, with the delay in distant cytokine mRNA up-regulation being significantly longer in the CDE model, which requires days rather than hours to develop. The relative timing of IL-1β mRNA appearance within the various organs is shown in Figure 3A and B for edematous and necrotizing pancreatitis, respectively, while systemic TNF-α mRNA expression is shown in Figure 3C and D.

Tissue Cytokine Protein Production. There was no immunoreactive IL-1β, IL-6, or TNF-α detectable within any tissues at baseline. Following the induction of both forms of pancreatitis, IL-1β and TNF-α became detectable in all organs except cardiac/skeletal muscle and kidney (all $P < 0.01$ vs control). IL-6 protein could only be detected within the pancreas ($P < 0.01$ vs control). The relative timing and degree of systemic elevation of IL-1β and TNF-α are shown in Figure 4. As was seen with mRNA production, the protein for these two cytokines was detectable within the pancreas prior to its detection in distant organs.

DISCUSSION

The current study demonstrates that the development of pancreatitis is associated with expression of proinflammatory cytokine message within the pancreas, increasing as pancreatitis severity increases. The expression of IL-1β and TNF-α mRNA coincides with the development of hyperamylasemia and histologic evidence of pancreatitis. This finding is not dependent upon animal strain, sex, age, or the model of pancreatitis utilized. The delayed expression of IL-6 mRNA within the pancreatic parenchyma is consistent with the previously demonstrated pattern of delayed IL-6 elevation in the serum during experimental and clinical pancreatitis (5–9). The protein product for all three cytokines was detected within

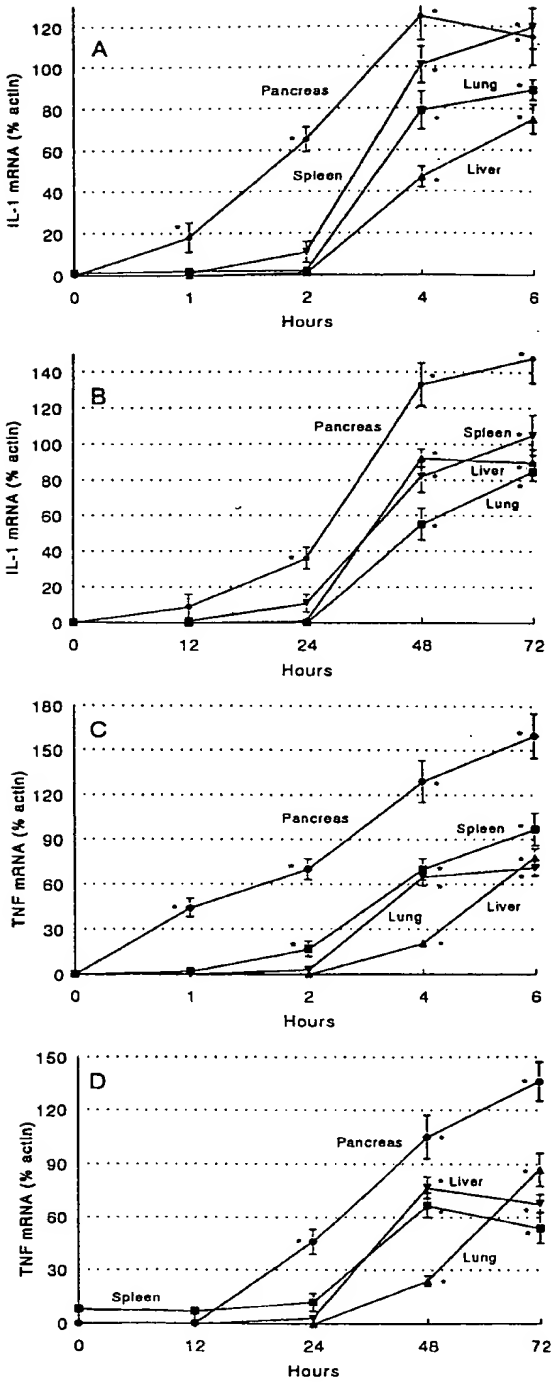

Fig 3. Relative timing of cytokine mRNA appearance in distant organs during the progression of acute pancreatitis. IL-1β or TNF-α to β-actin ratio is plotted during the development of edematous (A and C) or necrotizing (B and D) pancreatitis. Messenger RNA for both IL-1β and TNF-α was detectable within the pancreas several hours (edematous pancreatitis) or days (necrotizing pancreatitis) prior to it being detected in any other organ. Significant elevations ($P < 0.001$ vs time 0) in mRNA concentrations are denoted by an asterisk.

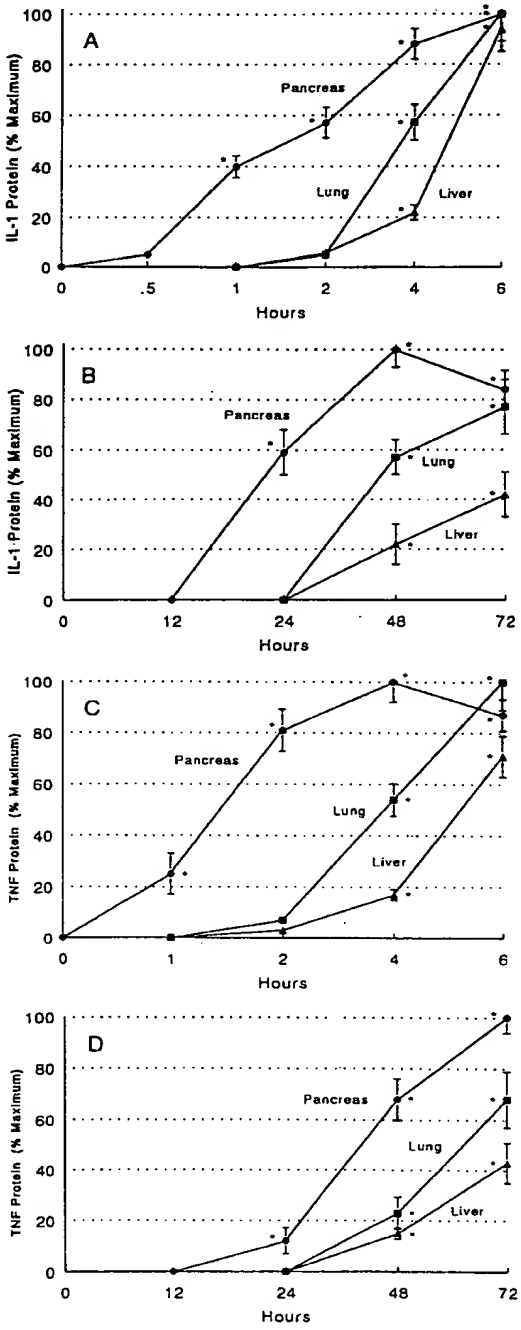

Fig 4. Timing of tissue IL-1β and TNF-α protein appearance by immunoblot. The development of pancreatitis was associated with the appearance of tissue cytokine levels where there was none detectable at baseline (all $P < 0.005$). Intrapancreatic cytokine protein was detectable several hours (edematous pancreatitis; A and C) or days (necrotizing pancreatitis; B and D) before it could be detected within the lung, liver, or spleen. All samples are standardized to serum protein concentration of 7.5 mg/dl and are shown relative to the density of pancreatic tissue blots for comparison.

pancreatic tissue shortly after their respective message, with each one increasing in direct correlation with worsening pancreatitis.

The differences in timing of cytokine production between the pancreas and distant organs is an important observation. IL-1β or TNF-α mRNA was not identified in a distant organ prior to its detection within the pancreas. Similarly, IL-1β and TNF-α protein was consistently found in the pancreas several hours prior to its detection within the tissues of the lung, liver, or within the systemic circulation.

The induction of multiorgan cytokine gene expression during experimental acute pancreatitis appears to be organ specific. All animals showed cytokine production within the lung, liver, and spleen. The kidney as well as cardiac and skeletal muscle, however, failed to demonstrate cytokine mRNA or protein during either model of pancreatitis. This would suggest that tissue rather than circulating leukocytes play a major role in specific organ cytokine production since not all perfused tissues demonstrate this phenomenon. This is consistent with previous reports that cytokine production has not been observed by leukocytes within the peripheral circulation during pancreatitis (22). The origination of these mediators in the lung, liver, and spleen, but not in other tissues, may be in part due to specific organ infiltration of activated leukocytes. Although adhesion molecule up-regulation (E-selectin, L-selectin, CD18), which has been demonstrated during the progression of pancreatitis (27) and is among the known properties of IL-1β and TNF-α (28–30), might be a possible mechanism for this specificity, it is improbable since the presence of circulating cytokines would likely cause generalized rather than specific tissue cytokine production. A more feasible scenario would be activation of the reticuloendothelial system by some circulating component present during acute pancreatitis since each distant tissue producing IL-1β and TNF-α has a large population of resident macrophages.

Proinflammatory cytokines have been implicated in the development of acute respiratory distress syndrome and multisystem organ failure associated with sepsis and pancreatitis (6, 31–34). The definitive demonstration of cytokine production in distant organs during the progression of pancreatitis provides further evidence that these mediators may play a large role in the progression of local pancreatic inflammation into a multiorgan inflammatory process. The signaling mechanism responsible for the delayed and specific manner in which the remote organs become involved remains to be elucidated.

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APPENDIX F

Amelioration of the Physiologic and Biochemical Changes of Acute Pancreatitis Using an Anti-TNF-α Polyclonal Antibody

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Tumor necrosis factor (TNF) is an inflammatory cytokine that may be an important mediator in the development of the systemic sequelae associated with severe acute pancreatitis. The purpose of this study was to determine whether the neutralization of TNF-α with a polyclonal antibody could ameliorate selected biochemical parameters of severe pancreatitis in a rat model. Pancreatitis was induced by an antegrade injection of artificial bile into the bile duct. Forty rats were randomized into 4 groups: no surgery (controls), saline infusion to bile duct (sham), placebo treatment in animals with pancreatitis (placebo + Px), and pretreatment with a polyclonal antibody (PAb) in animals with pancreatitis (PAb + Px). Serum TNF-α, amylase, calcium, hematocrit, glucose, and ascites volume were measured 2 hours after bile duct infusion. Pretreatment with the PAb produced a significant improvement in all parameters when compared with pancreatitis animals treated with placebo ($p < 0.001$). In addition, TNF-α, which was elevated in animals with pancreatitis, was reduced significantly in treated animals ($p < 0.001$). These results suggest that TNF-α may be an important mediator in the evolution of the systemic manifestations of severe acute pancreatitis.

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Tumor necrosis factor-α (TNFα) is a predominantly macrophage-derived cytokine that is thought to play a major role in mediating many of the pathophysiologic responses of an organism to injury or disease. The systemic manifestations of septic shock, which have been linked to the presence of elevated TNF-α levels, closely resemble those found in patients with severe acute pancreatitis. This has led us to investigate the role of TNF-α as a mediator of the systemic complications of severe acute pancreatitis. The clinical manifestations of complicated acute pancreatitis include hypovolemia, adult respiratory distress syndrome (ARDS), metabolic acidosis, disseminated intravascular coagulation, and electrolyte disturbances. Such complications can all be related to cellular responses, such as excessive neutrophil activation, enhanced capillary permeability, and direct cellular toxicity. These cellular events are linked to the production of TNF-α as demonstrated in septic shock syndrome.

We have previously reported detection of nanogram-per-milliliter quantities of TNF-α as early as 2 hours after the induction of acute pancreatitis in a rat model, which demonstrates many of the systemic manifestations of severe pancreatitis. In this rat model, TNF-α elevation was found to be independent of endotoxin [*1*]. The pathophysiologic manifestations of septic shock can be reproduced by the administration of TNF-α to animals [*2,3*] and can be blocked by anti-TNF-α antibodies [*4*]. Based on this, we hypothesized that if TNF-α is important in the pathogenesis of acute pancreatitis, as indicated by our previous studies, blocking it, then, should result in amelioration of the disease. The purpose of this study was to determine whether the neutralization of TNF-α with a polyclonal antibody (PAb) could ameliorate the physiologic, biochemical, and histologic changes of severe acute pancreatitis in a rat model.

MATERIALS AND METHODS

Animal model: Adult male Sprague Dawley rats (weight: 250 to 350 g) were used for all experiments to create a model of severe hemorrhagic pancreatitis. Anesthesia was induced with 10 mg/mL of intramuscular Ketamine (Ketastet, Aveco Co. Inc., IA) and Xylazine (Sigma Chemical, St. Louis, MO) at a concentration of 0.1 mL per 100 g and maintained with intravenous boluses of sodium pentobarbital. Indwelling polyethylene catheters (Becton Dickinson, Parsippany, NJ) were placed in the femoral artery and vein for the purposes of fluid and anesthetic administration, blood pressure monitoring, and blood sampling. On opening the abdomen, the

supraduodenal bile duct was isolated and cannulated with a polyethylene #10 catheter and connected to a Harvard pump (Sage Instruments, Cambridge, MA) to allow a constant pressure infusion of the artificial bile solution. This solution consisted of 75 mg sodium-taurocholic acid (derived from ox bile), 50 mg Keflin (Sigma Chemical, St. Louis, MO), and 45,000 IU of crystalline bovine trypsin (Sigma Chemicals, St. Louis, MO). Prior to infusion, the main pancreatic duct was ligated immediately proximal to the duodenum to ensure that the infused solution entered the pancreas. We have previously reported the use of this model to induce a severe hemorrhagic form of pancreatitis that is uniformly lethal and induces many of the physiologic, biochemical, and histologic changes that occur during severe hemorrhagic pancreatitis [1].

All experimental protocols received prior approval by the University of Tennessee Animal Care and Use Committee.

Experimental design: Forty rats were allocated to 1 of 4 groups (n = 10 each): (1) unoperated controls (control animals) were killed to provide biochemical and histologic baseline values; (2) sham controls (sham animals) received a 1-mL saline infusion to the bile duct; (3) placebo-treated pancreatitis animals (placebo + Px) received a 1-mL sterile saline injection through the penile vein immediately prior to induction of pancreatitis by a 1-mL infusion of artificial bile infusion into the bile duct; and (4) PAb-treated pancreatitis animals (PAb + Px) were pretreated with 1 mL of rabbit anti-TNF-α polyclonal antibody (Endogen, Boston, MA) via the penile vein 0.5 hours prior to the induction of acute pancreatitis. Blood pressure was monitored continuously throughout the procedure, and a minimum mean blood pressure of 70 mm Hg was maintained by intravenous fluid administration. Animals were monitored for 2 hours, at which point blood samples were taken for determination of TNF-α, amylase, calcium, glucose, and hematocrit levels. Ascites volume was measured, and pancreas and lung tissue was removed for histologic evaluation.

Analysis of plasma samples: All blood samples were centrifuged at 1,800 rpm for 10 minutes at 5°C. The plasma was removed using sterile pipettes. These samples were then immediately stored at −80°C until assayed.

TNF-α bioassay: A sensitive bioassay using the WEHI 164 subclone 13 cell line was utilized to detect TNF-α activity (permission for the use of the cells was granted by Dr. T. Espevik, Trondheim, Norway). This assay has been shown to have high specificity and sensitivity in the measurement of TNF-α [5,6]. One hundred microliters of WEHI cells (5×10^4 cells/well) with 0.5 μg/mL actinomycin D (Calbiochem, LaJolla, CA) was added to 96-well flat bottomed cell culture trays and incubated for 20 hours at 37°C with serial dilutions of plasma samples. The following day, 20 μL of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (Sigma, St. Louis, MO) at a concentration of 5 mg/mL in PBS was added to all wells and the plates incubated for a further 4 hours. Finally, 150 μL of media was removed, and 100 μL of 0.04 N HCl in isopropanol was added to each well to dissolve the purple formazan crystals. The absorbance at 550 nm was read the following day using a Bio-Rad (Richmond, CA) ELISA reader. TNF-α values were calculated using a standard curve prepared from human rTNF-α (specific activity 10^7 U/mg) and expressed in U/mL.

We have previously demonstrated that the cytotoxicity demonstrated in this assay was related to the activity of TNF by neutralizing all TNF-α positive samples with a polyclonal rabbit anti-TNF-α antibody that cross reacts with rat TNF-α followed by a repetition of the WEHI bioassay [1].

Serum amylase and calcium: Serum amylase was measured using a quantitative enzymatic assay according to the manufacturer's instructions (Sigma Diagnostics, No 575-UV, Sigma Chemicals, St. Louis, MO). Results are expressed in IU/L. Serum calcium was measured by atomic absorption spectrophotometry [7] and expressed in mg/dL.

Plasma glucose and hematocrit: Plasma glucose was measured immediately following sampling using a standard blood glucose monitoring system (Accu-Chek III, Boehringer Mannheim Corp., Indianapolis, IN). Hematocrit levels were determined by colorimetry following centrifugation of 0.5 mL of blood in a capillary tube and read from a standardized chart.

Measurement of ascites volume: Prior to the termination of the experiment, a plastic container with three absorbent gauze sponges was weighed. At the end of the procedure, the abdomen was opened, and the ascitic fluid was absorbed into the pre-weighed gauze sponges. After all the macroscopic ascites had been absorbed, the gauze sponges were wiped gently over the peritoneal surface to ensure complete absorption of the ascitic fluid. The plastic container with the ascites-soaked sponges was then reweighed. It was assumed that the specific gravity of the ascitic fluid was equal to that of water, and, thus, 1 g = 1 mL of ascites.

Tissue sampling and histologic examinations: The pancreas from treated and untreated rats was divided by a sagittal section into two halves. One half was fixed in 10% buffered formalin, embedded in paraffin, and stained by hematoxylin and eosin (H & E).

Morphometry: Three H & E-stained sections of each pancreas, taken at about 10-μ intervals, were analyzed. Each section was divided by a square lattice grid into 0.5 mm^2 squares to quantitate the histopathologic changes. Pathologic scoring was based on assigning numeric scores to describe pancreatic edema, vascular changes, fat necrosis, acinar necrosis, and pancreatic and peripancreatic calcification based on their extent and severity (**Table I**). A total score for each section was determined by adding scores of each square, then dividing by the number of squares examined. The final score of pancreatitis in an animal was the average of the pancreatitis score in the different levels.

Statistical analysis: Data deviated from the normal distribution, and thus all variables were ranked and analyzed using the nonparametric Kruskal-Wallis test. Significance was assigned when p was less than 0.05.

TABLE I
Histopathologic Scoring of Rat Pancreatitis

Edema
0 = Absent
0.5 = Focal expansion of the interlobar septae
1 = Diffuse expansion of the interlobar septae
Vascular
0 = Absent
0.5 = Congestion
1 = Congestion + focal interlobar or intralobar hemorrhage
1.5 = Multifocal diffuse hemorrhage
2 = Vascular fibrinoid necrosis or thrombosis
Fat necrosis
0 = Absent
0.5 = Focal dissolution of the interlobular or peripancreatic fat
1 = Diffuse dissolution of the interlobular or peripancreatic fat
Acinar necrosis
0 = Absent
0.5 = < 10% patchy necrosis of the edges of lobules
1 = 10%–30% patchy, peripheral necrosis of the lobules
1.5 = < 30% confluent lobular necrosis
2 = 30%–50% confluent lobular necrosis
2.5 = < 50% confluent lobular necrosis
3 = Microabscesses formation
Calcification
0 = Absent
0.5 = Focal in fat or acinar necrosis
1 = Diffuse in fat or acinar necrosis

RESULTS

Blood samples taken from normal control animals provided baseline measurements for TNF-α, amylase, calcium, hematocrit, and glucose levels, and ascites volume. Sham animals in which saline was infused into the pancreatic duct were not significantly different with respect to any of the biochemical or physiologic parameters measured in control animals. This indicated that neither the surgical procedure nor the infusion of saline into the bile duct resulted either in biochemical or TNF-α alterations. Placebo + Px animals manifested the biochemical and physiologic changes characteristic of acute pancreatitis, as demonstrated by marked hyperamylasemia, hypocalcemia, hyperglycemia, hemoconcentration, and the production of hemorrhagic ascites. These changes were significantly different from those of both control and sham animals for all parameters studied ($p < 0.001$). Pretreatment of animals with an anti-TNF-α PAb resulted in a significant improvement in all parameters studied (**Table II**).

TNF-α: Plasma TNF-α was undetectable in both control and sham animals, indicating that surgery itself does not contribute to elevated TNF-α levels. PAb + Px animals had significantly lower mean TNF-α plasma levels as compared with levels in the placebo + Px animals. However, TNF-α was not completely neutralized, and levels remained elevated at 35% of those levels seen in placebo + Px animals.

Amylase: Pretreatment with the PAb resulted in a significant reduction in the mean amylase level when compared with that in placebo-treated animals. Serum amylase levels in treated animals, however, remained statistically higher than in those animals in the control and sham groups ($p < 0.01$).

Calcium: Although the hypocalcemia noted in placebo + Px animals was significantly ameliorated in the PAb + Px group, serum calcium remained statistically different from that of control animals ($p < 0.05$).

Glucose: The hyperglycemia noted in placebo + Px animals was significantly improved by the administration of PAb. When both the control and sham animals were compared with the PAb + Px group, a statistical difference remained at the $p < 0.05$ level.

Hematocrit: A significant improvement in the mean hematocrit level was noted in PAb + Px animals when compared with the level in placebo-treated animals. However, no significant difference was noted when controls were compared with PAb animals ($p > 0.05$).

Ascites production: Ascites in control animals was not detectable. Animals pretreated with the PAb produced significantly less ascites than did placebo + Px animals.

Histologic evaluation: Control and sham-treated animals had normal gross pancreatic histology and, thus, were not examined by morphometry. Histologically, pancreatic sections from placebo + Px animals demonstrated extensive acinar necrosis, fat necrosis, calcium deposition, intrapancreatic hemorrhage, and vascular thrombosis. Lung sections demonstrated generalized congestion and areas of hemorrhage in the interalveolar septae. Morphometric examination of placebo + Px ($n = 5$) and PAb + Px animals ($n = 5$) revealed that despite amelioration of histologic changes seen in some areas of the pancreas in the PAb-treated animals, overall scoring was not statistically different (**Table III**).

COMMENTS

The results of this study demonstrated that the administration of an anti-TNF-α polyclonal antibody prior to the induction of acute pancreatitis resulted in a significant improvement in biochemical and physiologic derangements that are associated with severe acute pancreatitis. The validity of these results is further substantiated by the use of an animal model that demonstrates the biochemical and histologic features of severe acute pancreatitis.

The effect of TNF-α neutralization on the improvement in the systemic complications of acute pancreatitis is best explained by examining the known actions of TNF-α. TNF-α is thought to induce increased vascular permeability both by direct effects on vascular endothelium and by indirect mechanisms involving leukocytes. TNF-α promotes both the accumulation and activation of leukocytes in sites of inflammation [*8*]. Activated leukocytes are known to release several mediators under the influence of TNF-α that are known to augment capillary permeability. These mediators include platelet-activating factor [*9*], nitric oxide [*10*], phospholipase A_2 [*11–13*], and oxygen free radicals [*8*]. In addition, TNFα is known

TABLE II

Biochemical and Physiologic Differences in Study Animals (mean ± SEM)

Group	Amylase (IU/L)	Calcium (mg/dL)	Glucose (mg/dL)	Hematocrit (%)	Ascities (mL)	TNF-α (U/mL)
Control	1,400 ± 110	9.78 ± 0.53	104 ± 5	44 ± 0.1	0	0
Sham	1,267 ± 113	8.15 ± 0.21	106 ± 3	44 ± 0.7	1.00 ± 0.1	0
Placebo + Px	7,370 ± 643*	5.43 ± 0.44*	198 ± 15*	59 ± 0.9*	7.94 ± 0.5*	39.2 ± 5.3*
PAb + Px	2,988 ± 345†	8.15 ± 0.26†	118 ± 4*	47 ± 1.4*	5.43 ± 0.2†	13.7 ± 3.1†

Px = pancreatitis; PAb = polyclonal antibody.

* < 0.001 Px versus control and sham.

† p < 0.001 PAb + Px versus placebo + Px.

TABLE III

Results of Pathologic Scoring in Experimental Animals (mean ± SEM)

Group	Edema	Vascular Change	Fat Necrosis	Acinar Necrosis	Calcification	Total Score
Placebo + Px	0.8 ± 0.0	0.8 ± 0.1	0.4 ± 0.0	0.7 ± 0.1	0.3 ± 0.0	3.03 ± 0.19
PAb + Px	0.8 ± 0.0	0.9 ± 0.1	0.4 ± 0.0	0.8 ± 0.1	0.3 ± 0.0	3.14 ± 0.21

Px = pancreatitis; PAb = polyclonal antibody.

to up-regulate adhesion molecules, such as ELAM-1 [*14*] and ICAM-1 [*15*], on vascular endothelium. Up-regulated adhesion molecules contribute to capillary leakage and tissue damage by promoting leukocyte adherence and extravasation. Inhibition of capillary leakage by the neutralization of TNF-α may explain the improvement in ascites volume and hematocrit observed in treated animals.

Reversible hyperglycemia occurs in up to 50% of patients with acute pancreatitis. Infusions of TNF-α in rats result in early hyperglycemia, late hypoglycemia, and hypoinsulinemia [*16*]. This effect has been shown in animal studies to be dose dependent, with massive doses of TNF-α producing hypoglycemia and with lower doses inducing hyperglycemia [*17*]. Several mechanisms have been suggested to explain the effect of TNF-α on glucose metabolism. TNF-α is known to induce a state of insulin resistance in both hepatic and peripheral tissues [*18*]. In addition, TNF-α augments the production of catecholamines, glucagon, and cortisol, which, in turn, enhances gluconeogenesis and glycogenolysis [*19*]. Finally, TNF-α has a direct cytotoxic effect on pancreatic islet cells [*20*]. One or all of these mechanisms may account for the glucose intolerance observed in the animals with acute pancreatitis and, thus, explain the improvement following neutralization with the PAb.

Hyperamylasemia is a manifestation of pancreatic acinar injury. The etiology of acinar cell injury has not been clearly defined, although a number of mediators induced by TNF-α have been implicated. These mediators include activated proteolytic enzymes, phospholipase A_2, and oxygen free radicals, all of which can be measured in the serum, urine, and ascites of both animals and patients with acute pancreatitis. Neutralization of TNF-α may reduce the levels of these mediators and, thus, ameliorate the acinar cell damage and the hyperamylasemia that ensue.

Currently, the relationship between TNF-α and acute hypocalcemia is difficult to explain. The pathogenesis of hypocalcemia in acute pancreatitis or sepsis remains unknown, but is probably the end result of a number of processes: saponification of calcium salts in areas of fat necrosis, increased release of calcitonin, increased parathormone (PTH) release, or end-organ unresponsiveness to PTH [*21*]. One possibility is related to the presence of interleukin-1 (IL-1), which is known to be induced by TNF-α. IL-1 has been shown to strongly inhibit the renal and bone effects of the tumor-derived PTH-related peptide and thus to reduce its hypercalcemic, phosphaturic, and hypophosphatemic actions [*22*]. Indeed, bolus injections of recombinant human IL-1 into mice have been shown to induce significant hypocalcemia 3 hours after the first IL-1 injection [*23*]. Although IL-1 levels were not measured during the course of our experiments, separate studies have demonstrated the production of nanogram quantities of IL-1 in this model of acute pancreatitis (HPG *et al*, unpublished data, 1992).

Improvement in the biochemical markers of acute pancreatitis was also associated with significantly lower levels of plasma TNF-α, indicating that improvement correlated with the partial neutralization of TNF-α. Despite significant improvement in the PAb + Px-treated animals, when compared with placebo + Px animals, they remained statistically different from control animals for all parameters examined, except hematocrit levels. This may reflect inadequate dosage of the anti-TNF-α polyclonal antibody as suggested by the presence of TNF-α production in several treated animals. Alternatively, it may reflect the presence of other mediators that are not under the influence of TNF-α and whose production is

therefore not inhibited by the administration of an anti-TNF-α polyclonal antibody.

The administration of anti-TNF-α polyclonal antibody did not significantly affect the histologic picture of acute pancreatitis seen in these animals. Histologically, both treated and untreated animals demonstrated equivalent pancreatic changes (Table III). Considering the technique of induction of pancreatitis and the severity of the pancreatic changes in this model, this was not unexpected. This finding, however, confirms the important role of TNF-α as a systemic mediator of acute pancreatitis since PAb + Px animals had marked amelioration of systemic manifestation despite similar progression of local disease.

Taken together, our results suggest that TNF-α may play an important role as a systemic inflammatory mediator in acute pancreatitis and that TNF-α contributes to the systemic sequelae that are commonly observed in cases of severe acute pancreatitis. The ability of TNF-α blockade to ameliorate the biochemical manifestations of acute pancreatitis may have a significant implication in the management of humans with severe acute pancreatitis.

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DISCUSSION

Andrew L. Warshaw (Boston, MA): Although you used a traditional model, if the infusion pressure of bile salts is over 20 mm of mercury, the ducts will rupture, creating a very inhomogeneous chemical injury to the head of the gland, which often does not reach the tail of the gland. Your illustration showed this type of injury. What was the infusion pressure that you used?

Your finding of the effect of the antibody against TNF is of great interest. Ernst Klar, working in our laboratory, did the opposite experiment in which he gave TNF to animals with cerulein pancreatitis. Despite the dose or timing, adding TNF did not make the pancreatitis worse. Would you comment on the discrepancy?

Hani P. Grewal: Although we did not measure pressure, we were well aware of this factor. The saline-infused models did not show any evidence of pancreatitis, and the bile-infused rats showed a fairly even distribution of pancreatitis throughout the gland.

I cannot explain why TNF did not make the pancreatitis worse, in your studies. In our laboratory, when we injected TNF into rats that had acute pancreatitis, it made them significantly more unstable hemodynamically compared with animals that were pretreated with the antibody.

Donald E. Fry (Albuquerque, NM): Since TNF is clearly an autocrine and a paracrine signal more than an endocrine signal, the concentrations in serum or plasma probably reflect more the acuity of the insult rather than the total aggregate magnitude of the biologic perturbation.

What is the source of the polyclonal antibodies? Are they accumulated antibodies from animals that were given TNF and then the antibodies were harvested? What other components might be in this polyclonal antibody serum? TNF is of benefit to the host, and sustained anti-TNF therapy will probably ameliorate the acute response. Since these were only 2-hour experiments, longer term experiments may result in all the rats dying from nosocomial infections, since we are compromising an important local containment mechanism of the host.

Hani P. Grewal: Anti-TNF-α is derived from goat, and is an anti-mouse polyclonal antibody that has partial cross-reactivity with the rat TNF. We confirmed this by the neutralization of our positive samples with this anti-TNF antibody.

It would be very naive to consider that TNF is the sole mediator of the systemic manifestations of acute pancreatitis. The extrapolation from this research to the clinical situation would be very difficult. Patients with acute pancreatitis have numerous circulating endogenous mediators, although it is interesting that TNF does seem to be one of the early mediators of, and may be responsible for, triggering a cascade reaction that results in the production of several other mediators.

Frank G. Moody (Houston, TX): What is the role of inflammatory cytokines in the pathogenesis of pancreatitis? We found expression of interleukin 1β (IL-1β) at 2 hours in the ligated bile duct rat model. Have you performed any studies in an attempt to define if there is expression of TNF, IL-1 β, or IL-6, for example, since all three seem to play a role in these inflammatory responses?

Hani P. Grewal: Yes, we have, but measurement of cytokines in rats is difficult. The bio-assays are good, but others (such as ELISA) are not particularly reliable. We have measured messenger RNA (mRNA), which is an indirect measurement of cytokine secretion. We have primers for rat IL-6, and we have shown IL-6 elevations at the mRNA level. In humans, we have evidence from patients in intensive care units (ICUs) who have severe acute pancreatitis, showing differences in TNF, IL-1, IL-6, and IL-8 in those patients who have three or more Ransome criteria compared with those patients with mild pancreatitis with no evidence of systemic complications.

Paul Curley (Leeds, Great Britain): In your model, there is a very narrow band of treatment. We found that anti-TNF is detrimental with a fairly narrow treatment band compared with the dose at which they survive. Could you comment?

Hani P. Grewal: We used a soluble receptor, one that was supposedly very good for rat TNF, and found that all the rats died. Dr. Fry's comment addressed this. Complete neutralization of TNF and other cytokines is important in the body's inflammatory response against a particular insult. There must be a very fine line between reducing a detrimental inflammatory response and completely neutralizing it, which again is detrimental.

William P. Reed (Springfield, MA): Did you look at the effect of the timing of administration of your antibody on the development of pancreatitis in this model? Do the animals have to be pretreated in order for the antibody to be effective, or can the pancreatitis still be prevented if treatment is delayed for some interval of time after the insult?

Hani P. Grewal: We used a model with a less severe form of pancreatitis (mortality of 50% at 72 hours) and repeated these experiments. The results demonstrated a significant reduction in mortality in rats treated with the antibody, either 2 hours before or at induction of acute pancreatitis. We have not performed experiments to determine if there is any therapeutic benefit in giving this after the induction.